ROLE OF ESTROGEN RECEPTOR α AND OXIDATIVE STRESS RESPONSE PROTEINS IN THE CENTRAL NERVOUS SYSTEM

BY

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DISSERTATION

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ABSTRACT

Cellular metabolism results in the production of reactive oxygen species (ROS) as byproducts and can damage proteins, lipids, and DNA. Cells utilize a number of enzymes and small molecules to scavenge ROS and prevent and repair damage that has occurred. To better understand the role three of these proteins play in responding to ROS, we examined the activity of Cu/Zn superoxide dismutase, apurinic endonuclease, and estrogen receptor α in the central nervous system. Our studies demonstrate that oxidative protein damage in the spinal cord occurred prior to the appearance of motor symptoms in mice that express a mutant form of Cu/Zn superoxide dismutase and subsequently develop symptoms of amyotrophic lateral sclerosis. Additionally, we show increased apurinic endonuclease expression in the cerebral cortex of mice that had been ovariectomized, treated with 17β-estradiol, and then exposed to hypoxia. A decline in oxidative DNA damage occurred which we believe was the result of the 17β-estradiol-mediated increase in apurinic endonuclease expression. Finally, our work shows that estrogen receptor α protein expression in the cerebral cortex was stable in aging, hormonally intact females and this estrogen receptor α protein was functional. Altogether, these studies demonstrate the importance of understanding the impact of ROS-induced damage on human health and disease and provide insight to develop methods of prevention and treatment for neurodegenerative disorders.

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CHAPTER ONE:

Introduction

Molecular oxygen is a necessary component for the many oxidation-reduction reactions that sustain life. All aerobic cells utilize oxygen as an electron acceptor during oxidative phosphorylation. Specific free radical molecules known as reactive oxygen species (ROS) contain oxygen and are constantly produced in cells throughout the body. Examples of ROS include superoxide, which is the most abundant ROS, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite [1]. ROS have several important biological roles in the healthy cell including cellular signaling and host defense.

Biological roles of ROS

There is a misconception that ROS are only toxic byproducts of aerobic respiration but increasing evidence demonstrates that they are necessary for several physiological activities such as cellular signaling and phagocytosis. For example, hydrogen peroxide serves as a second messenger for MAP kinase activation and tyrosine phosphorylation [2-5]. Hydrogen peroxide is a highly diffusible molecule that can be quickly synthesized or broken down in response to stimuli, good qualities of a second messenger [2].

ROS participate in cytokine/growth factor action and secretion, ion transport, transcription, and apoptosis [6]. The targets of ROS involved in cellular signaling often include proteins such as thiols in G-proteins [7], transcription factors [8], and ion channels [9]. The specificity of targeted ROS signaling is based on several factors including the reduction potential of the target, the availability of other reactive species, a consensus sequence of redox active cysteine residues [10], or the placement of a particular cysteine residue [7]. ROS levels are regulated by the activity of their endogenous scavengers. These scavengers must not only inhibit ROS but must also modify and transport ROS involved in cellular communication [6]. Optimal NF-κB activation requires the controlled activity of glutathione [10]. Superoxide dismutase converts superoxide to hydrogen peroxide which can then form hydroxyl radical via the Fenton reaction. Thus regulation of ROS-activity occurs at three levels: 1) generation of species, 2) targeting, and 3) scavenger activity [6].

Another physiological role of ROS is in phagocytosis and immune function. Superoxide, hydrogen peroxide, and nitric oxide participate in host defense in neutrophils, eosinophils and

other polymorphonuclear phagocytes. Following activation via phosphorylation, NADPH oxidase produces superoxide in phagocytic cells. NADPH oxidase 2 produces hydrogen peroxide which then acts as a chemotactic factor for polymorphonuclear leukocytes [11]. Once at the appropriate region, ROS can also help to keep leukocytes and phagocytes in the area by inhibiting cell motility [12].

Oxidative stress

While ROS have biologically useful activities, the accumulation or abundance of ROS can lead to oxidative stress, a deleterious state in which the reduced intracellular environment is compromised. As superoxide is continually being generated during ATP production in mitochondria, the accumulation of ROS becomes a concern [13]. The mitochondrial respiratory chain, or electron transport chain, is composed of five protein complexes located within the inner membrane and arranged in order of increasing reduction potential [14]. During respiration electrons are passed from one complex to the next, but some electrons escape and participate in side reactions with molecular oxygen, partially reducing it to yield superoxide. Complexes I and III of the respiratory chain are the major sites of superoxide production from oxidative phosphorylation [15,16].

In addition to the large production of superoxide from the electron transport chain reactions of the inner mitochondrial membrane, hydrogen peroxide is produced in large quantities by monoamine oxidase in the outer membrane [13,14]. This enzyme catalyzes the oxidative deamination of biogenic amines which include the catecholamines, serotonin, and histamine. In dopaminergic neurons this enzyme is particularly active due to dopamine turnover [14]. Dopamine catabolism produces hydrogen peroxide as a byproduct and thus contributes to the development of Parkinson's disease. Thus, ROS are continually being generated both in the mitochondrial matrix and the cytosol.

ROS-induced damage

Cellular macromolecules including proteins, DNA, and lipids can become damaged during conditions of oxidative stress. While oxidation may serve regulatory functions in some cases, such as for the transcription factors NF-κB and AP-1 and in protein degradation, in other instances oxidation causes irreversible damage [17-19].

The amino acid residues histidine, lysine, arginine, proline, threonine, cysteine, aspartate, and tyrosine can all be modified by ROS. Examples of oxidative protein alterations include

carbonylation, thiol oxidation, and formation of isoaspartate and nitrotyrosine. Unlike the reversible oxidation of cysteine thiols, carbonylation of amino acid side chains in proteins is an example of irreversible ROS-induced protein damage. The proteasome [20] or autophagosomes [21] are utilized to degrade irreversibly oxidized macromolecules. Several oxidizing agents are involved in the formation of this damage including peroxynitrite, nitric oxide, and nitrite [22-25].

Oxidative DNA damage can occur as single base lesions, aldehyde adducts, strand breaks, or as the loss of a base, an apurinic site. These structural alterations are primarily mediated by hydroxyl radical, singlet oxygen, and hypochlorous acid as the main types of ROS that have been shown to interact with and cause damage to DNA [26,27]. ROS-induced DNA damage can include base pair mutations, mispairing, deletions, or insertions. Both nuclear and mitochondrial DNA are subject to ROS-induced damage, but the proximity of mitochondrial DNA and lack of repair proteins make it especially vulnerable to ROS-induced damage [13]. Single base, ROS-induced DNA damage is repaired via the short patch arm of the base excision repair pathway. Damage to three or more nucleotides is repaired via the long patch base excision repair pathway [28].

Lipids are also susceptible to ROS-induced damage. For example, peroxidation of polyunsaturated fatty acids such as arachidonatic acid can be induced by hydroxyl radical, peroxynitrite, and other potent oxidants [29]. When these oxidants react with lipids, hydrogen atoms are removed and the addition of oxygen to the lipid radical results in production of isoprostanes, stable prostaglandin-like products formed as a result of oxidative damage to polyunsaturated fatty acids [29,30]. The breakdown of lipid peroxidation produces malondialdehyde or other reactive aldehydes [31].

Biological implications of oxidative stress and ROS-induced damage

The biological implications of ROS dysregulation and damage include the development of cancer, inflammation, cardiovascular disease, neurodegenerative diseases, and many other conditions sensitive to the redox state of the cell. Oxidative stress is known to play an important role in the development and progression of cancer. In fact, cancer cells have increased levels of ROS, further enhancing the proliferation and genetic instability of tumor cells [32]. Oxidative stress has been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and stroke [33]. Similar types of

damage to proteins, DNA, and lipids are present in each of these disorders, indicating a similar origin and cascade of events that occur in each [34,35].

Oxidative stress response proteins

Cells have developed a system of enzymatic and non-enzymatic methods to restrict ROS production and utilization to appropriate subcellular locations, times, levels, molecular species, and for appropriate durations [12]. Many of the proteins involved in reducing ROS production, catabolizing ROS, repairing ROS-induced damage, or degrading macromolecules that cannot be repaired are known as antioxidants. These include superoxide dismutases, catalases, and enzymes of the glutathione and thioredoxin redox cycles. In addition to these antioxidant enzymes, small molecules including ascorbate, pyruvate, and α-ketoglutarate react with ROS non-enzymatically and can be recycled or replenished, giving them a ROS-buffering capacity [12].

Our laboratory previously identified five oxidative stress response proteins that associate with the DNA-bound estrogen receptor α (ERα) and influence estrogen responsiveness (Fig. 1.1; [36,37]). These oxidative stress response proteins include Cu/Zn superoxide dismutase (SOD1), thioredoxin (Trx), thioredoxin reductase (TrxR), protein disulfide isomerase (PDI), and apurinic endonuclease (Ape1). Each protein is responsible for reducing levels of ROS, maintaining a reduced intracellular environment, or repairing ROS-induced damage. For example, SOD1 is the first line of defense against ROS, and reduces superoxide to hydrogen peroxide [38]. Hydrogen peroxide is further reduced to water by cellular enzymes including catalase (Cat) and peroxiredoxins (Prx) [39]. Thioredoxin (Trx) is the major disulfide reductase and is responsible for reducing oxidized Prx to maintain its activity [40]. Thioredoxin reductase (TrxR) reduces Trx that has been oxidized by utilizing NADPH as its source of electrons. If oxidative stress occurs within the cell, proteins may become oxidized. When this occurs, Trx, TrxR, Ape1, and PDI help to reduce proteins to their native conformation [18,41].

One protein that cells utilize to reduce oxidative stress and repair damage is Ape1. This is a multifunctional protein that is necessary for base excision repair of DNA and is involved in the regulation of transcription factors via redox mechanisms [42,43]. The DNA repair function of Ape1 is especially important in the brain and neurons since these are post-mitotic cells and thus rely heavily on the base excision repair (BER) pathway to maintain DNA integrity [44-46]. The BER pathway repairs damage to single nucleotides and apurinic sites in DNA. BER is initiated by a DNA glycosylase when it

hydrolyzes the *N*-glycosylic bond between the sugar molecule and DNA base leaving an apurinic site that is recognized by Ape1 [47]. Next, Ape1 hydrolyzes the phosphodiester bond in the DNA backbone 5` to the apurinic site and DNA polymerase β then inserts an intact nucleotide [48-52]. DNA ligase seals the backbone and thus completes BER [53]. The brain is an especially vulnerable organ for ROS-induced damage because it is the most metabolically active organ in the body. This high metabolic activity results in greater ROS production and oxidative stress.

Ape1 is also important in reducing oxidized proteins and maintaining transcription factors in a reduced, active state. Studies have demonstrated that Ape1 facilitates DNA binding of p53, NFκB, Fos, Jun, and HIF1α [54-57]. When cysteine residues become oxidized, transcription factors are no longer able to recognize or bind to their respective DNA sequences. Once this occurs, Ape1 can directly reduce oxidized transcription factors and also assists other reducing proteins such as Trx and glutathione by binding to select transcription factors, enhancing reduction of their cysteine residues, and increasing their DNA binding activity [19]. Thus, Ape1 is involved in transcriptional regulation as well as DNA repair and maintenance of protein structure and function.

Another oxidative stress response protein I have examined in my studies is SOD1. While three forms of superoxide dismutase exist two are cellular, SOD1 and SOD2, and a third is extracellular, SOD3 [38]. SOD1 is mainly considered a cytoplasmic protein and utilizes copper and zinc at its catalytic center to reduce superoxide to hydrogen peroxide. SOD1 responds to a variety of chemical and biologic stressors including UVB and irradiation [58-60], hydrogen peroxide [61], ozone [62], and nitric oxide [63]. The importance of SOD1 to respond to oxidative stimuli is highlighted in studies in which motor neurons from SOD1 deficient mice exhibit increased susceptibility to oxidative stress [64]. Furthermore, multiple mutations in the SOD1 gene are known to contribute to the development of amyotrophic lateral sclerosis (ALS; [65,66]).

Amyotrophic Lateral Sclerosis

While it is known that oxidative stress plays a role in stroke, Parkinson's disease, Alzheimer's disease, and ALS, alterations in SOD1 expression and activity have also been linked to neurodegenerative diseases such as familial ALS and Alzheimer's disease [46,67,68]. Familial ALS is linked to mutations in SOD1, many of which are missense mutations and some that render the enzyme nearly inactive [65,66]. It has been suggested that upper motor neurons in the cerebral cortex and lower motor neurons in the brainstem and spinal cord are damaged by

oxidative stress resulting in degeneration of these neurons [69,70]. Symptoms of ALS include muscle weakness, difficulty walking, slurred speech, and loss of dexterity. Paralysis and death generally occur within three to five years from the onset of these symptoms due to the progressive atrophy of affected muscles [71,72].

Some ALS-associated mutations interfere with the ability of SOD1 to dismutate superoxide to hydrogen peroxide and can result in the accumulation of superoxide [73-75]. Excess superoxide can then react with nitric oxide to form peroxynitrite ($OONO^-$) which is capable of nitrosylating tyrosine residues and altering protein structure and function (Fig. 1.2; [33]). Because ROS are short lived and difficult to measure directly, the results of the damage they inflict are often used as a measure of the relative amount of oxidative stress present.

While the aggregation of SOD1 in ALS and its symptoms are well documented, the development, progression, and upstream processes that contribute to SOD1 aggregation and ALS symptoms are not well understood. Based on the knowledge that some SOD1 mutations associated with ALS prevent the enzyme from dismutating superoxide and that the accumulation of superoxide can result in nitrosylation of tyrosine residues, I hypothesized that oxidative protein damage might increase in transgenic mice that express a mutant form of SOD1 compared with wild type mice.

SOD1 and neuroprotection

Previous studies have demonstrated that SOD1 protects the brain from ischemia/reperfusion injury or other neurodegenerative event. Work conducted by Pak Chan and coworkers using transgenic mice that overexpress SOD1 shows that infarct size and brain edema are decreased in transgenic mice compared to nontransgenic mice [76,77]. Furthermore, the loss of SOD1 in mice increases infarct volume, brain swelling and neuronal cell death following ischemia [78]. Additional studies have demonstrated that SOD1 is important in repairing injury to motor neurons and that this is dependent on SOD1 gene dosage [64]. These studies reveal the importance of SOD1 activity in preventing or repairing damage following neuronal injury.

Additional studies have provided insight to define how SOD1 mediates its neuroprotective effects. Chan and coworkers observed that levels of active glutathione were significantly increased following transient ischemia in the penumbra region of transgenic mice that over express SOD1 compared to non-transgenic mice [76,77]. These studies demonstrate a role for superoxide radicals in the pathogenesis of reperfusion injury and indicates that SOD1

may help maintain other antioxidant levels and prevent oxidative stress-induced injury and neuronal death after ischemia and reperfusion. Increased SOD1 levels and activity could also eliminate the formation of peroxynitrite since the lack of superoxide radicals to react with nitric oxide would prevent the generation of peroxynitrite.

An additional method by which SOD1 reduces the infarct volume and damage following ischemia is by the reduction of neuronal cell death. Studies have demonstrated that SOD1 prevents the early release of cytrochrome c from mitochondria and inhibits the mitochondrial pathway of caspase activation [79,80]. SOD1 further inhibits neuronal cell death by increasing Akt phosphorylation and decreasing ERK1/2 phosphorylation [81,82]. Finally, SOD1 is indirectly involved in DNA repair by helping to maintain levels of the DNA repair protein Ape1 following ischemia [83,84].

Ape1 and neuroprotection

Cells also utilize Ape1 as a means of neuroprotection under ischemic or hypoxic conditions. Studies in mice demonstrate that following ischemia, a decline in Ape1 expression occurs and this leads to increased DNA damage and cell death [83,85,86]. However, if Ape1 expression is maintained or elevated, cell death, DNA damage, and cerebral infarct volume resulting from middle cerebral artery (MCA) occlusion are significantly reduced [84,87-89]. Additional studies in which Ape1 was overexpressed in mice exposed to MCA occlusion demonstrate that infarct volume, DNA damage, and apoptosis are reduced compared to animals with normal Ape1 expression [87].

Both the DNA repair and redox functions of Ape1 are beneficial for its neuroprotective activities. In fact, not only does Ape1 recognize and repair apurinic sites that result from ROS, but it also enhances the activity of the enzyme which it follows in the base excision repair pathway, 8-oxoguanine-DNA glycosylase [48,90-92]. In this way, Ape1 increases the rate at which repair of oxidative lesions in DNA occurs.

E_2 and neuroprotection

Not only are antioxidants and proteins that maintain or repair macromolecules involved in neuroprotection, but 17β-estradiol (E_2) is also known to mediate neuroprotective effects. Some of the best known work in this area is from studies by Wise and coworkers in which an MCA occlusion model was used to demonstrate that treatment of ovariectomized female rodents with physiological levels of E_2 had significantly reduced levels of infarct damage and cell death [93-

96]. However, neuroprotection was only observed when E_2 was administered prior to artery occlusion [97]. Additional studies demonstrated that E_2 protection occurred in estrogen receptor β (ERβ) but not in ERα null mice that have been subjected to MCA occlusion, indicating that ERα is necessary for E_2-mediated neuroprotection [98,99]. Another group has determined that neuronal ERα is the cellular target of E_2 in the brain [100]. Thus, E_2 mediates it effects through neuronal ERα in the brain to limit ischemic damage in the cerebral cortex.

Menopause is characterized by the decreased production of ovarian hormones and this decline in hormones corresponds to an increase in stroke risk for women. Therefore, it has been suggested that this decline contributes to the increased stroke risk that women develop following menopause [93,101]. In fact, a woman's risk of suffering a stroke doubles in the ten years following menopause [102,103]. From studies in rodents and humans, a "timing hypothesis" has been proposed and states that women achieve the greatest benefit from hormone replacement therapy when it is initiated during the menopausal transition or early postmenopause [104-107].

The most compelling example of the importance of time between cessation of ovarian function and initiation of hormone replacement therapy is the Women's Health Initiative. While initial studies were halted after invasive breast cancer was shown to be increased in the combined estrogen/progesterone arm of the study, further reanalysis revealed that when E_2 replacement begins within ten years after the onset of menopause, women experience reduced rates of coronary heart disease and mortality [106,107].

In addition to the influence of E_2 on neural tissue, E_2 has profound effects on uterine tissue [108,109]. For example, E_2 regulates gene expression and influences cellular proliferation, remodeling, and angiogenesis in the uterus [110]. Not only are genes that are involved in endometrial remodeling regulated by E_2, but oxidative stress response proteins are also altered by E_2 treatment in the uterus [111] demonstrating that E_2 differentially influences the expression of oxidative stress response proteins and emphasizes the need for selective estrogen receptor modulators that influence these and other antioxidant enzymes favorably in order to avoid detrimental repercussions.

Because of the constant ROS production by cellular metabolism, oxidative stress response proteins are essential in order to maintain cellular homeostasis, and this is especially important in the brain where metabolic activity is greatest. The contribution of even a single protein such as SOD1 demonstrates that a reduction in its expression or activity can have far-reaching consequences including increased oxidative stress, protein damage, and ultimately

result in the development of a fatal disease state. Likewise, Ape1 is necessary to maintain protein and DNA integrity, a loss of which can lead to alteration of gene expression, DNA fragmentation, and cell death. Altogether, the impact of ERα and oxidative stress response proteins on cellular macromolecules and pathways demonstrates their necessity for the survival of cells, and their influence on human health and disease.

Figures and Figure Legends

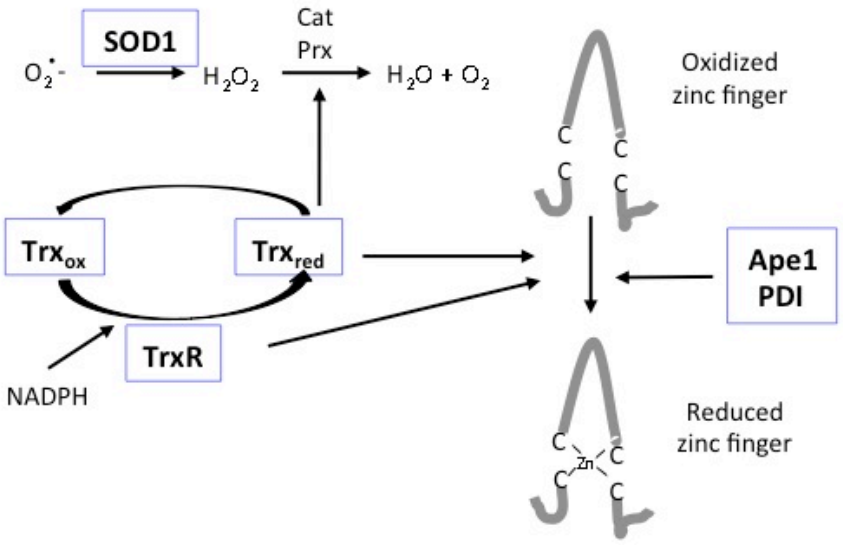

Fig. 1.1 Oxidative stress response proteins help maintain protein structure and function. Cu/Zn superoxide dismutase (SOD1) reduces superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) which is then converted to water by peroxiredoxins (Prx) and catalase (Cat). Reduced thioredoxin (Trx) activates Prx and thioredoxin reductase (TrxR) converts oxidized Trx to reduced Trx using NADPH as a cofactor. Trx, TrxR, apurinic endonuclease (Ape1), and protein disulfide isomerase (PDI) assist in converting oxidized zinc finger proteins to reduced proteins so they remain functional.

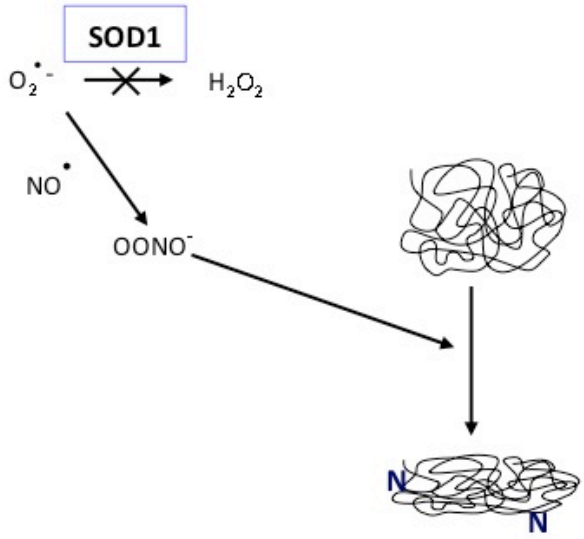

Fig. 1.2 The accumulation of superoxide leads to the formation of nitrotyrosine residues in proteins. When superoxide ($O_2^{\bullet -}$) accumulates it reacts with nitric oxide ($NO^{\bullet}$) and can produce peroxynitrite ($OONO^-$). Peroxynitrite nitrosylates tyrosine residues in proteins and can alter their structure and function.

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CHAPTER TWO:

Increased Oxidative Protein Damage in a Rodent Model of Amyotrophic Lateral Sclerosis

Abstract

Amyotrophic lateral sclerosis (ALS) is a degenerative neurological disease that leads to paralysis and death within three to five years of the appearance of motor dysfunction. A common feature of sporadic and familial ALS is the formation of protein aggregates in spinal cord motor neurons. Although the underlying process involved in formation of these aggregates is not well understood, it has been suggested that oxidative damage to motor neurons may be involved in both forms of ALS. To better understand the process involved in protein aggregate formation, we examined spinal cord sections from transgenic mice that express wild type Cu/Zn superoxide dismutase 1 (WT SOD1) or a G85R SOD1 mutant protein, each of which is fused to yellow fluorescent protein. We showed that protein damage and protein aggregate formation predated the onset of motor neuron dysfunction in transgenic mice that expressed the mutant G85R SOD1 protein, but not in transgenic mice that expressed the WT SOD1 protein.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects the upper motor neurons in the cerebral cortex as well as the lower motor neurons of the brainstem and spinal cord [1,2]. Sporadic and inherited forms of this disease cause progressive atrophy of muscles and paralysis. Sporadic cases of ALS account for the majority of patients and are not associated with inherited genetic alterations [2]. Approximately 25% of patients have familial ALS which results from mutations in the oxidative stress response protein copper zinc superoxide dismutase (SOD1) [3,4].

More than 160 mutations in SOD1 have been identified to date that lead to ALS [5], the large majority of which are missense substitutions scattered throughout the five exons of the gene. The majority of mutant SOD1-associated familial ALS is inherited in an autosomal dominant manner so that patients are typically heterozygous, expressing both the wild-type and mutant forms of SOD1 [2,6]. A common feature of both sporadic and familial ALS patients is the accumulation of insoluble protein aggregates [7] indicating that protein misfolding and aggregation are shared pathological features. Furthermore, sporadic and familial ALS are nearly indistinguishable clinically and pathologically [3,4], leading to the hope that strategies for treating familial ALS may translate to at least a subset of sporadic cases [8].

It has been hypothesized that the misfolding of mutant SOD1 protein leads to formation of protein aggregates [2,4,6,9]. Wild-type SOD1 functions as a homodimer in which one copper and one zinc ion in each monomer are critical for its activity of converting superoxide to hydrogen peroxide. However some SOD1 mutations, such as those involved in coordinating the copper or zinc ion, render the enzyme nearly inactive [10]. When this occurs, superoxide accumulates in the cell and damage to macromolecules including DNA, lipids, and proteins can occur.

Mutation of glycine at amino acid 85 to arginine, which is located in the metal binding region, causes a misfolded SOD1 mutant protein (G85R SOD1). G85R SOD1 is associated with ALS in humans and causes motor neuron death and paralysis in transgenic mice [3,11,12]. Earlier studies indicated that free radical generation is increased in the presence of some SOD1 mutants [13,14] and that oxidative damage is increased in familial and sporadic ALS patients [15]. To better understand the development of ALS and its progression, we examined effects of the G85R SOD1 mutation on oxidative protein damage by examining the expression and

localization of nitrosylated tyrosine residues, which have been used as an indicator of protein damage.

2. Materials and Methods

2.1. Transgenic mice

Transgenic mice were derived as described by Wang et al [12]. Briefly, a plasmid containing the human SOD1 gene [16] was modified to express the entire enhanced YFP gene (Clontech). A G85R mutation in exon IV of the SOD1 gene was introduced by oligonucleotide-directed PCR mutagenesis of a restriction fragment, which was then substituted for the wild-type fragment. This fragment was sequenced to verify that only the desired nucleotide change was present. The final constructs were digested to produce DNA sequences that were injected into mouse embryos (C57BL/6J x SJL/J F2). Transgenic founder mice were identified by PCR amplification of DNA extracted from tail biopsies. Lines were maintained by periodic crossing with nontransgenic C57BL/6J x SJL/J F1 mice from Jackson Laboratory (Bar Harbor, ME).

2.2. Immunofluorescent staining

20 μm spinal cord sections from 1, 2.5, or 5 month old G85R SOD1 mice and 3 month old WT SOD1 mice were obtained from Arthur Horwich (Yale University, New Haven, CT). The sections were dried at room temperature, dipped in PBS to remove O.C.T. compound (Sakura, Torrance, CA), post-fixed in 2% paraformaldehyde for 15 min, and washed 2x with PBS. 0.1% Trypsin-EDTA with 0.1% $CaCl_2$ was used for antigen retrieval by incubating slides at 37°C for 30 min. Slides were then washed 2x with PBS, permeabilized in PBS with 0.5% Triton X-100 for 10 min, washed 2x with PBS, and incubated in blocking solution (PBS with 5% normal donkey serum and 0.1% Triton X-100) for 30-60 min. A nitrotyrosine-specific antibody (1:200, 06-284, Millipore, Billerica, MA), was diluted in blocking solution and incubated on slides for 2 h. Slides were washed 3x with PBS, incubated with DyLight 649-conjugated donkey anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted in blocking solution for 1 h in the dark at room temperature, washed 3x with PBS, and stained with DAPI to identify nuclei. Lipofucsin-like autofluorescence was reduced by incubating slides in 50 mM ammonium acetate and 10 mM copper sulfate for 1 h. Slides were dipped in H_2O then PBS and mounted with Pro-Long Gold antifade mounting media.

2.3. Image collection and quantitation

All images were obtained either with a 10x objective or 40x oil-immersion objective using a Leica DM 4000 B confocal microscope and Leica TCS SPE system and Application Suite Advanced Fluorescence software (Leica Microsystems, Inc., Bannockburn, IL). Detector gain and offset, laser power, and bandwidth of emission collection were kept constant for all sections in each experiment and adjusted so that images had a full range of pixel intensities (0–255) and saturation was minimized. Images from three independent experiments were collected to examine nitrotyrosylated proteins over time.

Image Pro Plus software (Media Cybernetics, Bethesda, MD) was used for quantitative immunofluorescent analysis of nitrotyrosine staining in spinal cord sections. Analysis included 10-32 fields in 5-10 spinal cord sections from 3 month old WT- and 1, 2.5 and 5 month old G85R SOD1-expressing mice. The perimeter and area were adjusted so that only specifically stained aggregates were detected and recorded in a script designed to analyze individual images in an entire z-stack. Data were exported into Excel and the mean area of specifically stained aggregates in each z-stack image was quantitated for each treatment. SAS 9.1 Basic Statistics (SAS Institute, Cary, NC) was used for statistical analysis. Data were combined and are presented as the mean ± SEM.

3. Results

3.1. SOD1 aggregation in disease progression

Immunofluorescent microscopy was used to examine the spinal cords of transgenic mice that expressed wild type (WT SOD1) or mutant (G85R SOD1) SOD1, each of which was fused to YFP, to examine formation of protein aggregates during disease progression. Accumulation of insoluble protein aggregates occurs in lower motor neurons within the anterior horn of the spinal cord [2,12]. The approximate location of the gray matter, where motor neurons reside, is outlined in a lumber spinal cord section from a WT SOD1 mouse (Fig. 2.1). At 3 months of age, low levels of SOD1 fluorescence were detected in WT SOD1 mice. By 1 month of age, SOD1 protein aggregates were present in spinal cords from G85R SOD1 mice. At 2.5 months of age, the mutant G85R SOD1 aggregates were larger and more numerous. By 5 months, the spinal cords from G85R SOD1 mice had many smaller, less intense fluorescent aggregates scattered throughout the gray matter and even the surrounding white matter.

When visualized at higher magnification, SOD1 expression was nearly undetectable in WT SOD1 mice (Fig. 2.2A). In contrast, 1 month old G85R SOD1 mice had discrete aggregates. The aggregates were significantly larger in 2.5 month-old G85R SOD1 mice. At 5 months of age, the aggregates were diminished in size and fluorescent intensity. The same image collection settings were used for samples from WT and G85R SOD1 mice.

3.2. Protein damage in disease progression

SOD1 is the primary enzyme responsible for converting the free radical superoxide ($O_2\bullet^-$) to less damaging hydrogen peroxide (H_2O_2) [17] (Fig. 2.2B). Other cellular enzymes then reduce hydrogen peroxide to water. When a mutation in the metal binding region occurs, such as G85R, SOD1 can no longer efficiently dismutate superoxide to hydrogen peroxide [11,12,18], which can lead to the accumulation of superoxide. Physiological levels of nitric oxide (NO•) react with excess superoxide to form the potent peroxynitrite ($ONOO^-$) that is capable of nitrating protein tyrosine residues [4]. Nitrotyrosine can dramatically alter protein structure and function, leading to protein aggregation.

In order to determine whether protein nitrosylation was increased in G85R SOD1 mice, spinal cord sections were stained using a nitrotyrosine-specific antibody to detect oxidative protein damage. As shown in Fig. 2.2C, no nitrotyrosine staining was observed in 3 month old mice that expressed WT SOD1. However nitrotyrosine staining was already apparent in 1 month old G85R SOD1 mice. Interestingly, the nitrotyrosine staining overlapped with the aggregated G85R SOD1. At 2.5 months, the nitrotyrosine staining increased as G85R SOD1 aggregates increased in size and number. At 5 months, nitrotyrosine staining was still observed although it was more dispersed, much like the G85R SOD1 protein.

Image analysis software was used to quantitate the average area of the nitrotyrosine damage over time and indicated that at 1 month, G85R SOD1 mice had larger areas of protein damage than 3 month old WT SOD1 mice (Fig. 2.3D). There was a significant increase in the area of protein damage in 2.5 month old G85R SOD1 mice compared with transgenic mice that expressed WT SOD1 while the average area of damage decreased in 5 month old mice.

4. Discussion

The formation of insoluble protein aggregates in spinal cord motor neurons is a hallmark of both sporadic and familial ALS and yet the underlying steps involved in the onset and progression of this disease are largely unknown. We have now shown that nitrotyrosine, an

indicator of oxidative protein damage, accumulates as disease progresses but then diminishes in area and fluorescence at the end stage of disease.

Our studies of G85R SOD1 transgenic mice have demonstrated that protein nitration was present in spinal cord motor neurons by 1 month of age and overlapped with G85R SOD1 protein aggregation. Since nitration of even a single tyrosine residue can result in significant alterations in protein structure and function [4], it is likely that protein aggregation occurred following nitrotyrosine formation. Although G85R SOD1 mice did not exhibit motor symptoms at 1 month of age, insoluble SOD1 aggregates and nitrotyrosine protein damage were already obvious in the anterior horn of the spinal cord.

By 2.5 months, the age when motor symptoms begin to appear (Maria Nagy, personal communication), protein aggregation and nitrotyrosine damage were widespread and abundant. The average area of oxidative protein damage significantly increased in 2.5 month old mice that expressed G85R SOD1 compared with mice that expressed WT SOD1. At 5 months, the age when paralysis occurred, protein aggregates and nitrotyrosine staining decreased in size and fluorescence intensity. This decreased aggregate size may have resulted from motor neuron cell death and release of their contents into the extracellular environment [6,19,20].

ALS in humans is often characterized by rapid disease progression in which paralysis and death occur within 3 to 5 years from the onset of symptoms [7,21]. Thus, by the time a patient presents with motor symptoms consistent with ALS, it is likely that spinal cord motor neurons already have extensive aggregates and oxidative protein damage. In fact, increased tyrosine nitration has been observed in spinal cord tissue from both sporadic and familial ALS patients [15], consistent with our findings of accumulated protein aggregation and nitrosylation in spinal cords of mice expressing G85R SOD1.

Our studies demonstrate that aggregate formation and oxidative protein damage are early features of familial ALS that occur prior to the onset of motor symptoms. This work extends our understanding of the onset and progression of protein damage in familial ALS and may also inform us about the early events in sporadic ALS as well since the symptoms and pathology are so similar. Nitration of proteins at early stages of other neurodegenerative diseases has also been observed [4], indicating a possible common pathway of damage among diseases in which protein aggregation occurs.

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Figures and Figure Legends

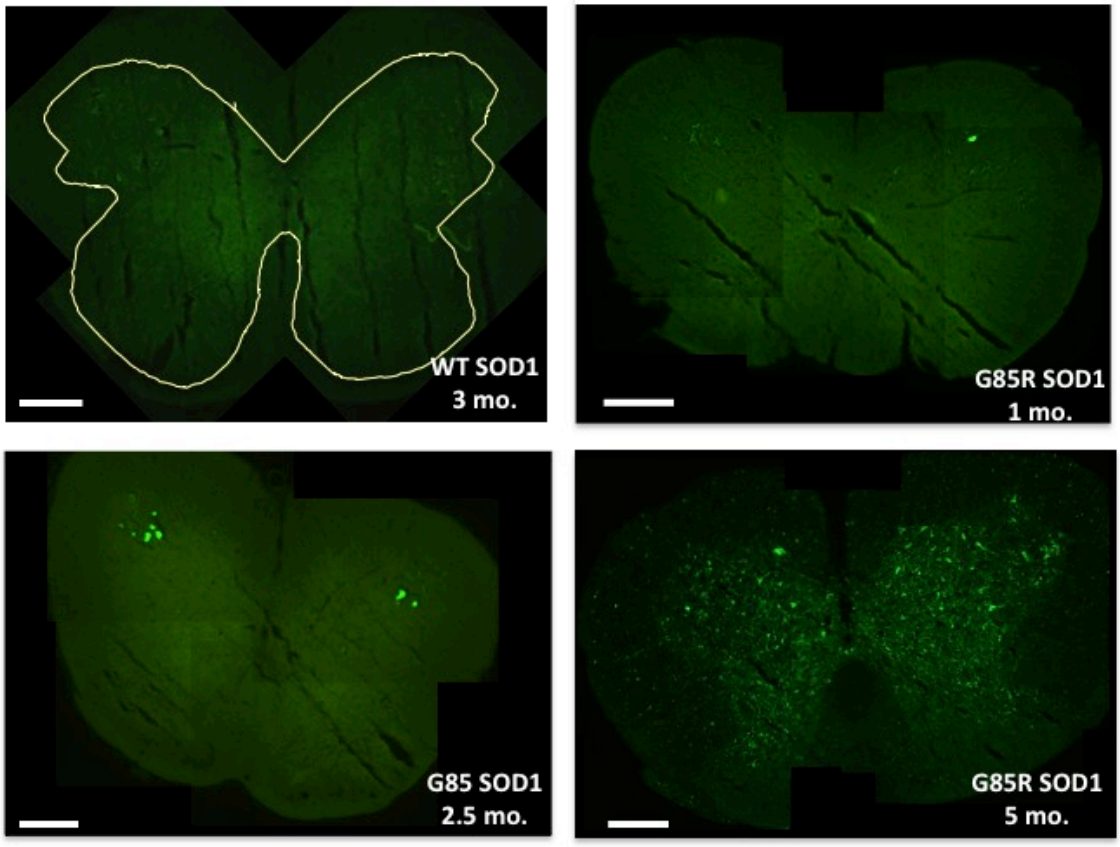

Fig. 2.1 Low magnification view of spinal cord sections from transgenic mice expressing wild-type or mutant SOD1. Immunofluorescence was used to detect YFP-tagged WT SOD1 or G85R SOD1. Spinal cords were harvested from mice at the indicated ages, sectioned with a cryostat, and imaged. Scale bars indicate 500 μm.

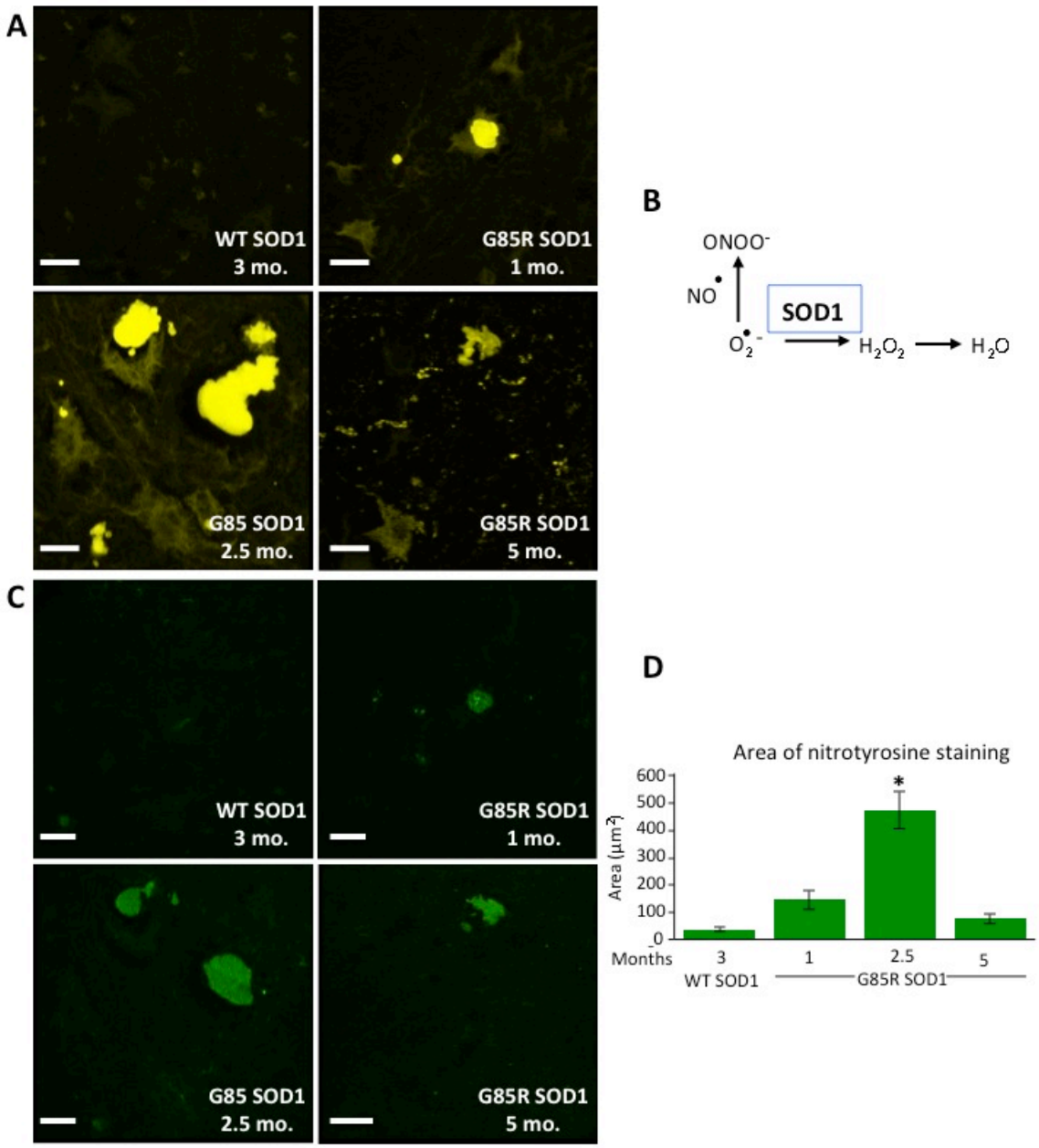

Fig. 2.2 High magnification view of spinal cord sections from transgenic mice expressing WT or mutant SOD1. (A) Immunofluorescent microscopy was used to detect YFP-tagged WT or G85R SOD1 from transgenic mice at the indicated ages. (B) During normal cellular metabolism SOD1 converts superoxide ($O_2\bullet^-$) to hydrogen peroxide (H_2O_2), which is then converted to water by other cellular enzymes. If superoxide accumulates, it can react with nitric oxide (NO•) to form peroxynitrite ($ONOO^-$), which can in turn lead to nitration of tyrosine residues. (C) A nitrotyrosine-specific antibody and immunofluorescence were used to detect nitrosylated proteins. Scale bars indicate 25 μm. (D) Image analysis of nitrotyrosine staining area in spinal cord sections from WT SOD1 or G85R SOD1 transgenic mice at the indicated age. Data from 7-22 images/animal were combined and are expressed as the mean area ± SEM. A significant increase in nitrotyrosine area occurred at 2.5 months compared with 3 month WT (*$p < 0.05$).

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CHAPTER THREE:

17β-Estradiol Increases Expression of the Oxidative Stress Response and DNA Repair Protein Apurinic Endonuclease (Ape1) in the Cerebral Cortex of Female Mice Following Hypoxia

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Abstract

While it is well established that 17β-estradiol (E_2) protects the rodent brain from ischemia-induced damage, it has been unclear how this neuroprotective effect is mediated. Interestingly, convincing evidence has also demonstrated that maintaining or increasing the expression of the oxidative stress response and DNA repair protein apurinic endonuclease 1 (Ape1) is instrumental in reducing ischemia-induced damage in the brain. Since E_2 increases expression of the oxidative stress response proteins Cu/Zn superoxide dismutase and thioredoxin in the brain, we hypothesized that E_2 may also increase Ape1 expression and that this E_2-induced expression of Ape1 may help to mediate the neuroprotective effects of E_2 in the brain. To test this hypothesis, we utilized three model systems including primary cortical neurons, brain slice cultures, and whole animals. Although estrogen receptor α and Ape1 were expressed in primary cortical neurons, E_2 did not alter Ape1 expression in these cells. However, immunofluorescent staining and quantitative Western blot analysis demonstrated that estrogen receptor α and Ape1 were expressed in the nuclei of cortical neurons in brain slice cultures and that E_2 increased Ape1 expression in the cerebral cortex of these cultures. Furthermore, Ape1 expression was increased and oxidative DNA damage was decreased in the cerebral cortices of ovariectomized female C57Bl/6J mice that had been treated with E_2 and exposed to hypoxia. Taken together, our studies demonstrate that the neuronal microenvironment may be required for increased Ape1 expression and that E_2 enhances expression of Ape1 and reduces oxidative DNA damage, which may in turn help to reduce ischemia-induced damage in the cerebral cortex and mediate the neuroprotective effects of E_2.

1. Introduction

The human brain utilizes 20% of the oxygen consumed, but accounts for only 2% of total body mass, making it the most metabolically active organ in the human body [1]. Because reactive oxygen species (ROS) are produced as byproducts of normal cellular metabolism, the massive consumption of oxygen by the brain can lead to substantial ROS production. ROS play a role in cellular signaling such as MAP kinase activation and tyrosine phosphorylation [2-5]. However, if not effectively dissipated, ROS can accumulate and the ability of the cell to maintain a reduced intracellular environment is compromised, which can result in oxidative stress and damage to resident proteins, lipids, and DNA.

Cells rely on a variety of proteins to dissipate ROS, reduce oxidative stress, and, if damage does occur, to repair ROS-induced damage to cellular macromolecules. The oxidative stress response protein apurinic endonuclease (Ape1) is a multifunctional protein involved in DNA repair and redox regulation. Ape1 is the primary mammalian endonuclease and plays an essential role in repairing the most common DNA lesions, apurinic and 8-hydroxydeoxyguanosine (8-OHG) sites [6-11]. In addition to its role in DNA repair, Ape1 is required for the reduction of oxidized cellular proteins and is especially important in maintaining numerous transcription factors in a reduced, active state [12-15]. Thus, Ape1 is required to maintain DNA integrity and protein structure and function in the brain.

Ape1 is also instrumental in repairing cellular damage caused after blood vessel occlusion as might occur during a stroke [16-18]. While the hypoxia resulting from blood vessel occlusion is deleterious, reoxygenation can be even more damaging as the oxygen supply is reestablished and ROS production rapidly escalates [19-21]. Studies in rodents have demonstrated that Ape1 levels decline following middle cerebral artery (MCA) occlusion leading to DNA damage and cell death in the infarct region [16,22,23]. However, by simply maintaining Ape1 levels, cell death and DNA damage can be reduced [16,17]. Furthermore, if Ape1 is overexpressed, DNA damage and cerebral infarct volume resulting from MCA occlusion is significantly reduced [18].

While best known for its role in female fertility, the steroid hormone 17β-estradiol (E_2) also alters the expression of proteins involved in oxidative stress response, anti-inflammatory processes, and programmed cell death in the brain [24-35]. A number of laboratories have demonstrated that E_2 diminishes neuronal injury associated with cerebral ischemia and brain

trauma in rodents [36-40]. This E_2-induced neuroprotection is most apparent in the cerebral cortex, which is particularly vulnerable to ischemia-induced injury [17,18,24-27,41]. An elegant series of studies by Wise and coworkers demonstrated that exposing ovariectomized female rodents to physiological levels of E_2 reduces the infarct volume and cell death that occurs following MCA occlusion [24-27], but that this neuroprotective effect was only observed when the E_2 was administered prior to artery occlusion [28]. Furthermore, the fact that E_2 pretreatment decreases infarct volume in estrogen receptor β (ERβ), but not in estrogen receptor α (ERα) null mice that have been subjected to MCA occlusion, demonstrates that ERα, not ERβ, is involved in this E_2-mediated neuroprotection [29,30].

We previously demonstrated that E_2 increases expression of the oxidative stress response protein Cu/Zn superoxide dismutase in brain slice cultures and that this E_2-induced expression helps to limit protein and DNA damage of cerebral cortical neurons [42]. From these studies we hypothesized that E_2 might also increase Ape1 expression in the brain and that this E_2-induced expression of Ape1 could help to protect the cerebral cortex from hypoxia-induced cell damage.

2. Materials and Methods

2.1. Mice

C57BL/6J breeding pairs for the generation of mouse pups for isolation of primary cortical neurons and brain slice cultures and female mice (12-15 weeks) were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12 h light/dark schedule with access to water and food ad libitum. Ovariectomized mice were maintained on phytoestrogen-free chow. Primary cortical neurons were prepared from P0 female mouse pups, non-neuronal cultures for the preparation of conditioned media were prepared from P0-P3 female mouse pups, and brain slice cultures were prepared from P7 to P9 day old female pups. Female pups were identified by the smaller anogenital distance and the absence of an adjacent pigmented region. All procedures were performed in accordance with guidelines of the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee and Division of Animal Resources.

2.2. Primary cortical neurons

The isolation of primary cortical neurons was performed as described [43] with the following modifications. Conditioned plating medium (Neurobasal-A with 0.5 mM GlutaMAX, B27 supplement without antioxidants [Life Technologies, Grand Island, NY], and antibiotics)

and conditioned maintenance medium (Neurobasal-A with 0.5 mM GlutaMAX, custom-formulated media supplement [Table 1], and antibiotics) were prepared by incubating non-neuronal cultures with media for 24 h. Cortical pieces were triturated in conditioned plating media, cells were counted and seeded on poly-D-lysine coated 60 mm petri dishes at 115,000 cells/cm^2 or on poly-D-lysine and fibronectin pre-coated German glass coverslips (EMS, Hatfield, PA) at 50,000 cells/cm^2. Neurons were incubated overnight and the conditioned plating media was removed and replaced with conditioned maintenance media. Primary cortical neurons were treated with 1 μM cytosine ß-D-arabinofuranoside once a week to reduce glial cell proliferation. Media was removed from the primary cortical neurons and replaced with conditioned maintenance media twice a week. Ethanol or 20 nM E_2 was added 24 h prior to cell harvest. All cells were maintained in a 5% CO_2 incubator at 36°C.

2.3. Brain slice cultures

Brain slice cultures were prepared essentially as described [44] with modifications. Pups were decapitated and whole brains were quickly removed, mounted, and sectioned with a Leica VT1200 vibratome (Leica Microsystems, Nussloch, Germany). 300 μm coronal sections were sliced into chilled slicing solution (1.25 mM NaH_2PO_4, 2.5 mM KCl, 10 mM $MgSO_4$, 0.5 mM $CaCl_2$, 234 mM sucrose, 11 mM glucose and 26 mM $NaHCO_3$). Slices were immediately placed on a sterile Millicell culture plate insert (Millipore, Billerica, MA) in each well of a chilled 12-well plate. Excess slicing solution was removed following sectioning and replaced with 1.2 ml of Neurobasal-A medium (Gibco, Carlsbad, CA) containing 0.5 mM GlutaMAX (Gibco), antibiotics (penicillin, streptomycin, and gentamycin) and 10% charcoal dextran-treated fetal bovine serum with ethanol or 20 nM E_2. Slices were maintained in a 5% CO_2 incubator at 36 °C for 24 h.

2.4. Ovariectomy

12-15 week old female mice were anesthetized by inhalation of 4% isoflurane, bilaterally ovariectomized, and implanted subcutaneously with silastic tubing (0.062 in/0.125 in, inner/outer diameter, 1 in length; Dow Corning, Midland, MI) plugged at both ends with medical adhesive (Dow Corning). Tubing contained 35 μl cottonseed oil (vehicle) or 35 μl of 180 μg/ml E_2 in cottonseed oil, which produces a low physiological level of circulating E_2 (~25 pg/ml) [29] that is equivalent to estrous levels of E_2 in mice [45-47].

2.5. Hypoxia treatment

7 days after ovariectomy and implantation of silastic tubing, oil- and E_2-treated mice were placed in cages inside a hypoxia chamber (BioSpherix, Lacona, NY) that was equilibrated to 7% O_2 and 93% N_2 for 3 h, conditions which have been shown to induce substantial changes in mRNA expression [48]. Oxygen concentration was monitored continuously throughout the experiments. Oil- and E_2-treated mice were also maintained at normoxic conditions for 3 h. After hypoxia, animals were allowed to reoxygenate for 1 h (RT-PCR) or 3 h (protein expression and DNA damage). Following hypoxia and re-oxygenation, all animals were anesthetized with isoflurane, decapitated, and cerebral cortices were harvested for quantitative real time-PCR, immunofluorescent staining, Western blot, or DNA damage analyses.

2.6. Western blot analysis

Primary neurons, brain slice cultures, or cerebral cortical hemispheres were combined with 400, 250, or 800 μl RIPA buffer (Thermo Scientific, Rockford, IL), respectively, with 1x Protease Inhibitor Cocktail (Sigma) and homogenized for 10 seconds at high speed with a Pro Homogenizer (ProScientific Inc., Oxford, CT). The buffer was adjusted to 400 mM NaCl with 5 M NaCl, placed on ice, and vortexed every 5 min for 15 min. The extract was spun at 20,800 x g in a 4°C microfuge, the supernatant was removed, and protein assays were performed with bicinchoninic acid using BSA as the protein standard (Thermo Scientific). Whole cell lysates (25 μg) were loaded onto each lane of a denaturing gel and fractionated. Proteins were transferred to a nitrocellulose membrane and blots were probed with an Ape1- (1:2000, ab194, Abcam Inc., Cambridge, MA), β-tubulin- (1:5000, sc9104, Santa Cruz Biotechnologies, Santa Cruz, CA) or α-tubulin- (1:100,000, T6199, Sigma) specific antibody followed by a secondary antibody covalently linked to infrared fluorophores (IRDye 800CW donkey anti-mouse IgG [1:5,000, LI-COR Biosciences, Lincoln, NE] or IRDye 800CW donkey anti-rabbit IgG [1:5,000, LI-COR Biosciences]). The membranes were scanned with an Odyssey infrared imager (LI-COR Biosciences) to quantitate the level of protein present. The integrated intensity function with the automated median background correction method was used since this system provides a wide dynamic range and reduces error to improve quantitative accuracy [49,50].

2.7. Immunofluorescence imaging

Brain slices from cultures and whole animals were rinsed 2x with PBS, fixed in PBS with 4% formaldehyde for 1 h, washed 3x with PBS, permeabilized with PBS containing 1% Triton

X-100 for 30 min, and incubated in blocking solution (PBS with 0.05% Tween-20 and 5% normal donkey serum) for 1–2 h. Slices were then incubated in blocking solution with an ERα-, (1:600, ab31312, Abcam Inc.), Ape1- (1:100, sc9919, Santa Cruz Biotechnologies), or NeuN- (1:500, MAB377, Millipore, Temecula, CA) specific antibody for 2 h at room temperature. Slices were washed 3x with PBS containing 0.1% Tween-20 (PBS-T) and incubated with DyLight 549-, 649- or 488-conjugated anti-rabbit, anti-goat or anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), respectively, for 1 h in the dark at room temperature, washed 3x with PBS-T, incubated with DAPI nucleic acid stain for 30 min at room temperature, washed 3x with PBS, and mounted with Pro-Long Gold antifade reagent (Life Technologies). 4′,6-diamidino-2-phenylindole (DAPI) co-staining was included with each treatment to identify nuclei and ensure that similar numbers of cells were present. Control slices, which had not been exposed to primary antibody, were processed in parallel.

Primary cortical neurons were stained as described for the brain slices with the following modifications. After primary neurons were rinsed 2x with PBS, they were fixed in PBS with 4% formaldehyde for 15 min, washed 3x with PBS, permeabilized with PBS containing 0.5% Triton X-100 for 10 min, and incubated in blocking solution for 30 min. DAPI staining time was reduced to 10 min.

2.8. Image collection and quantitation

All images were obtained with a 40x oil-immersion objective using the Leica DM 4000 B confocal microscope and Leica TCS SPE system and Application Suite Advanced Fluorescence software (Leica Microsystems, Inc., Bannockburn, IL). Detector gain and offset, laser power, and bandwidth of emission collection were kept constant for all treatments in each experiment and adjusted so that images had a full range of pixel intensities (0–255) and saturation was minimized. Images from 8 independent experiments were collected from primary cortical neurons to examine effect of E_2 on Ape1 expression.

Image Pro Plus software (Media Cybernetics, Bethesda, MD) was used for quantitative immunofluorescent analysis of Ape1 staining in brain slice cultures. Analysis included 477 (ethanol) or 742 (E_2) z-stack images from 6 (ethanol) or 7 (E_2) individual brain slice cultures. The perimeter and area were adjusted so that only specifically stained cells were detected and recorded in a script designed to analyze the individual images in an entire z-stack. Data were

exported into Excel and the mean density/intensity of specifically stained cells in each z-stack image was quantitated for each treatment.

2.9. RT-PCR

Total RNA was isolated from mouse cortices using RNAqueous reagents (Ambion, Life Technologies, Austin, TX) according to the manufacturer's instructions. RNA concentrations were measured and cDNA was synthesized using the iScript kit (Bio-Rad, Hercules, CA) as described by the manufacturer. 1μl of cDNA was combined with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), forward and reverse primers for HIF3α (5'-GGACTCAGACTCAGGCTACAG-3' and 5'- TCAGGAAGTGGACGCAGATG-3') and VefgA (5'- GGCTGCTGTAACGATGAAG-3' and 5'- TCTGCTGTGCTGTAGGAAG-3') and real-time PCR was carried out using a Bio-Rad iQ5 multicolor Real-Time PCR Detection System. Standard curves were created using cDNA equivalents of 0.125, 1.25 and 12.5 ng of RNA and were run in triplicate with each primer set for each experiment.

2.10. 8-hydroxydeoxyguanosine quantitation

Genomic DNA was isolated by digesting each cerebral cortex in 500 μl of DNA isolation buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% SDS, 1 mg/ml proteinase K [Sigma], 200 μg/ml RNase A [Sigma]) overnight at 50°C in a rotating incubator. After tissue digestion, 500 μL of phenol-chloroform-isoamyl alcohol (25:24:1) was added and samples were vortexed and centrifuged at 15,000 x g for 5 min at room temperature. The top, aqueous phase was transferred to a fresh tube. DNA was precipitated, resuspended in Tris-EDTA and 8-_hydroxydeoxyguanosine (8-OHG) oxidative DNA damage was quantitated using the OxiSelect Oxidative DNA Damage ELISA kit (Cell Biolabs, San Diego, CA). A 96 well plate was coated with 8-OHG conjugate overnight. Sample DNA was denatured, digested, and treated with alkaline phosphatase. Individual samples or 8-OHG standards were added to the coated plate and 8-OHG antibody was added to each well for 1 hr. Wells were rinsed with wash buffer 3x and secondary antibody was added. After 1 hr, wells were washed 3x and the substrate solution was added. Stop solution was added to each well after the color had developed and the absorbance was read at 450 nm using a SpectraMax Plus 384 plate reader.

2.11. Statistics

Combined data are expressed as the mean ± SEM. SAS version 9.2 (SAS Institute Inc., Cary, NC) was used for statistical analysis. Ape1 protein expression in brain slice cultures was

analyzed using Student's *t*-test. Statistical analyses of HIF3α and VegfA mRNA expression, Ape1 protein expression, and 8-OHG levels among all groups of animals were compared using a two-way analysis of variance (ANOVA). A p value of <0.05 was considered statistically significant (95% confidence interval).

3. Results

3.1. Ape1 expression in primary cortical neurons

Since neuronal ERα is essential for E_2-mediated neuroprotection [29,30,36] and the cerebral cortex is particularly susceptible to ischemia-induced damage [17,18,24-27,41], we examined the expression of ERα and Ape1 in primary cultures of cortical neurons. Primary neurons were isolated from the cerebral cortices of newborn C57Bl/6J female mouse pups and cultured for 9 or 10 days in vitro. When an ERα antibody was preincubated with purified ERα and used in immunofluorescence assays, no staining was detected (data not shown). In contrast, antibody that had not been preincubated with receptor detected robust ERα staining, thus confirming the specificity of the antibody (Fig. 3.1A). Quantitative real-time PCR demonstrated that ERα transcripts were present in primary neurons that had been cultured for 9 days (data not shown). Furthermore, immunofluorescent staining demonstrated that the expression of Ape1 was quite robust in these cells as well suggesting that they might be an appropriate model system to study the potential effects of E_2 on Ape1 expression.

When primary cerebral cortical neurons were treated with ethanol vehicle or 20 nM E_2 for 24 h, no changes in Ape1 expression were detected using Western blot (Fig. 3.1B) or immunofluorescence (Fig. 3.1C) analyses. Ape1 staining was confined to the nuclear compartment of the primary neurons (Fig. 3.1C, compare Ape1 and NeuN staining). Varying the concentration (10-100 nM) or time (3-48 h) of E_2 treatment did not alter Ape1 expression (data not shown). Even after eliminating the superoxide dismutase, catalase, corticosterone, and progesterone from the B27 media supplement, which are typically used for primary neuronal cultures (Table 1), we were still unable to detect an E_2-induced expression of Ape1. Thus, in spite of the fact that ERα was present and would presumably have been able to respond to E_2 treatment, no changes in Ape1 expression were observed suggesting that the primary neurons may require other cell types and/or the organizational features and structural architecture present in the intact brain.

3.2. Ape1 expression in brain slice cultures

To more closely recapitulate the neuronal environment present in the brain, we used brain slice cultures, which maintain much of the spatial architecture and many of the organizational features and local synaptic connections present in the brain [44]. Since the cellular environment can be carefully defined and manipulated, brain slice cultures have been used extensively to study electrophysiological properties, angiogenesis, dendritic growth, neural cell migration, and the responsiveness of neural cells to various drugs and treatments including E_2 [42,51-55].

Brain slice cultures were prepared from 7-9 day old C57Bl/6J mouse pups. Immunofluorescent staining was used to characterize the expression of Ape1 and ERα in the cerebral cortex. In order to decrease variation and limit bias, the same regions of the cerebral cortex (Fig. 3.2A, red boxed regions) were examined in each of the experiments described herein. Ape1 was expressed in the nuclei of cerebral cortical neurons as shown by co-staining with DAPI and the neuronal marker NeuN (Fig. 3.2B and C). Similarly, ERα was expressed in the nuclei of cerebral cortical neurons (Fig. 3.2D). In contrast, no staining was observed when the Ape1- or ERα-specific antibody was omitted (data not shown). The co-expression of ERα and Ape1 in the nuclei of the same cerebral cortical neurons was evident when the ERα and Ape1 channels were merged (Fig. 3.2E).

When brain slice cultures were treated with ethanol or 20 nM E_2 for 24 h and stained with an Ape1-specific antibody, Ape1 staining was observed in the absence of E_2, but when brain slice cultures were treated with E_2, Ape1 staining was increased (Fig. 3.3A). Immunofluorescence image analysis of ethanol- or E_2-treated brain slice cultures demonstrated that Ape1 expression was significantly increased in the cerebral cortex when cultures were treated with E_2 compared to cultures that were treated with ethanol vehicle (Fig. 3.3B).

Whole cell extracts were prepared from brain slice cultures that had been treated with ethanol or 20 nM E_2 for 24 h and Western blot analysis was performed to examine the effect of E_2 on Ape1 expression using another independent method (Fig. 3.3C). Combined data from 8 individual brain slice cultures demonstrated that E_2 significantly increased Ape1 expression (Fig. 3.3D). These findings confirmed that E_2 increased Ape1 expression in cortical neurons and demonstrated that brain slice cultures provide a valuable model system to study estrogen action in the brain.

3.3. Effect of hypoxia on Ape1 expression and DNA damage in the cerebral cortex of mice

Previous studies have demonstrated that significantly less neural damage is observed when ovariectomized female rodents are treated with E_2 and subjected to MCA occlusion than when animals have been treated with oil [24-27]. Thus, MCA occlusion experiments have been extremely informative in demonstrating the neuroprotective effects of E_2. However, because the infarct region contains dead or dying cells using this experimental paradigm, the amount of information that can be gathered from these cells is limited. Thus, rather than use an artery occlusion method, we examined the effect of hypoxia, a hallmark of ischemia, in ovariectomized female mice that had or had not been treated with E_2.

C57Bl/6J female mice were ovariectomized and silastic tubing containing oil or E_2 was implanted. After 7 days, a time when E_2-mediated neuroprotection is observed in rodents [25,28-30,56,57], mice were placed in a hypoxia chamber that was equilibrated to 7% oxygen to mimic the decrease in oxygen that can occur with ischemia. The animals were allowed to recover in normoxic conditions and then sacrificed. Control mice that had not been exposed to hypoxia were processed in parallel.

Previous studies have shown that hypoxia induces the expression of several genes in the brain including HIF3α and VegfA [58-62]. Quantitative real-time PCR demonstrated that when animals were exposed to hypoxia for 3 h and allowed to recover for 1 h, HIF3α and VegfA transcript levels were significantly increased (Figs. 3.4A and B, respectively).

Immunofluorescent staining of the cerebral cortex from ovariectomized female mice that had been treated with oil or E_2 for 7 days, exposed to hypoxia for 3 h, and allowed to recover to normoxic conditions for 3 h indicated that Ape1 (Fig. 3.5A) and ERα (Fig. 3.5B) were present in the nuclei of the cerebral cortical neurons. Whole cell extracts were prepared from cerebral cortices to determine whether E_2 altered Ape1 expression during hypoxia using Western blot analysis (Fig. 3.6A). As seen in Fig. 3.6B, a modest but significant increase in Ape1 expression was observed when mice were treated with E_2 and exposed to hypoxia. Thus, E_2 treatment and hypoxia together increased Ape1 expression in the cerebral cortex of ovariectomized female mice.

Guanine is particularly susceptible to oxidation and is the most common target of oxidative DNA damage. 8-hydroxydeoxyguanosine (8-OHG) mispairs with adenine and results in a guanine to thymine transversion and perturbation in DNA conformation [63]. To determine

whether E_2 altered DNA damage during hypoxia, the level of 8-OHG was examined. Ovariectomized female mice were treated with oil or E_2 for 7 days, exposed to hypoxia for 3 h, recovered at normoxic conditions for 3 h, and then sacrificed. Control mice that had not been exposed to hypoxia were processed in parallel. Genomic DNA was isolated from cerebral cortices and 8-OHG levels was measured and reported as the normalized mean ± SEM (Fig. 3.6C). E_2 significantly reduced 8-OHG levels in the cerebral cortex of mice that had been exposed to hypoxia.

4. Discussion

Although it has been known for some time that E_2 decreases ischemia-induced damage in the rodent brain, the mechanisms mediating this neuroprotective effect have remained unclear [24-27]. We now provide evidence for a link between E_2 treatment, Ape1 expression, and neuroprotection. Our studies demonstrate that E_2 increased Ape1 expression in the cerebral cortex of brain slice cultures and that combined hypoxia and E_2 treatment increased Ape1 expression and decreased 8-OHG oxidative DNA damage in the mouse cerebral cortex.

4.1. Ape1-induced neuroprotection

Previous studies have convincingly demonstrated that Ape1 protects the brain from ischemia-induced injury by decreasing DNA damage and cell death [16-18,22,23]. One way Ape1 might protect the cerebral cortex from ischemia-induced damage is by repairing DNA. In fact, results from our studies demonstrate that when E_2 was administered prior to a hypoxic event, the levels of the oxidative DNA damage marker 8-OHG were significantly decreased.

Ape1 is an essential enzyme in the base excision repair pathway. Ape1 recognizes and repairs apurinic sites and also enhances the activity of 8-oxoguanine-DNA glycosylase, the enzyme responsible for removing 8-OHG lesions [6,9-11]. Furthermore, Ape1 activity in base excision repair is especially vital in post-mitotic cells such as neurons that rely predominantly on this pathway to maintain DNA integrity [64-66]. When Ape1 expression declines after MCA occlusion, extensive DNA and cellular damage is observed [16,17]. However, if Ape1 expression is maintained or elevated, the number of DNA lesions significantly decline [17,18,67,68]. Our work demonstrates that even the modest elevation of Ape1 protein expression observed in mice that had been treated with E_2 and then exposed to hypoxia is sufficient to decrease oxidative DNA damage. Thus, the E_2-induced increase in Ape1 expression may help to protect the cerebral cortex from ischemia-induced DNA damage.

Another mechanism by which Ape1 can reduce ischemic injury is by maintaining numerous proteins in an active, reduced state. When ROS accumulate, cellular proteins can become oxidized and are no longer functional. Ape1 reduces a number of transcription factors including p53, NFκB, Fos, Jun, and HIF1α [12-15]. The ability of these transcription factors to modulate expression of numerous genes could have widespread effects on gene expression, cellular function, and neuronal survival [7,69]. Thus, Ape1 is required to maintain protein structure and function and preserve cellular homeostasis that is critical in the central nervous system. The overall importance of Ape1 is evident in the embryonic death of Ape1 null mice [70,71].

4.2. Combined data from the three models

At first glance the findings from our cell-based, brain slice, and whole animal studies may seem inconsistent. However, a more careful inspection highlights the fact that each model system provides clues about the requirements for Ape1 expression in the cerebral cortex. The fact that no increase in Ape1 expression was observed in primary cortical neurons seems contradictory to the brain slice and whole animal studies where E_2 increased Ape1 expression. However, it seems plausible that the failure of E_2 to alter Ape1 expression in primary neurons might be attributed to the lack of architectural and organizational features present in the brain microenvironment and/or the absence of other cells such as astroglia, microglia, or oligodendrocytes. Recently, E_2 has been shown to increase glial cell release of growth factors that promote neuronal survival [72]. The presence of these other cells and/or organizational features in the brain slice cultures may be required for E_2-induced expression of Ape1 as observed in the brain slice cultures. When the neuronal environment was more closely recapitulated in brain slice cultures, E_2 treatment was sufficient to enhance Ape1 expression. However, both hypoxia and E_2 treatment were required for increased Ape1 expression in the whole animal. If one considers that brain slice cultures are exposed to significant stress during harvesting and slicing, this stress combined with E_2 treatment may be sufficient to increase Ape1 expression in brain slice cultures and supports the idea that E_2 alone may not be sufficient to increase Ape1 expression.

4.3. Role of E_2-induced proteins in neuroprotection

Although our studies focused on the E_2-induced expression of a single oxidative stress response protein, Ape1, E_2 enhances the expression of other oxidative stress response proteins

including Cu/Zn superoxide dismutase and thioredoxin, which are known to reduce ischemia-induced damage [42,73-75]. Superoxide dismutase reduces superoxide levels and decreases protein and DNA damage in neurons [42]. Thioredoxin, like Ape1, reduces oxidized cellular proteins and enhances ischemia-induced neuronal survival [76,77]. Overexpression of Ape1, Cu/Zn superoxide dismutase, or thioredoxin confers a neuroprotective effect following MCA occlusion [18,77,78]. Furthermore, intravenous administration of thioredoxin in mice following ischemia reduces infarct volume, protein damage, and cell death [79]. Together these studies emphasize the important role that oxidative stress response proteins have, as a whole, in sustaining cell viability after an ischemic event [16,17,23,77].

Although an E_2-mediated increase in Bcl-2 expression has also been implicated in inhibiting apoptosis after an ischemic event [34,35], it seems unlikely that simply limiting apoptosis in neurons with extensive DNA damage would be beneficial. However, if the E_2-mediated increase in Bcl-2 expression was coupled with increased expression of oxidative stress response and DNA repair proteins, together these proteins could reduce protein and DNA damage and enhance neuronal cell survival.

In addition to reducing oxidative stress and apoptosis by mediating the induction of oxidative stress response proteins and Bcl-2 in the brain, E_2 attenuates inflammation by reducing pro-inflammatory molecules, decreases cytokine and chemokine release following administration of a neuroinflammatory endotoxin, and reduces microglial activation and peripheral monocyte recruitment [31-33].

While it is clear that ERα plays a role in E_2-mediated neuroprotection [29,30], other studies have implicated a role for ERβ in neuroprotection [80]. Both ERα and ERβ are involved in E_2-mediated neurogenesis in the subventricular zone, which generates neural stem cells that migrate to the site of injury [81]. In addition, estrogen binding sites in the mitochondria and plasma membranes have also been implicated in conferring estrogen responsiveness [82,83].

4.4. Biological relevance of E_2-induced Ape1 expression

The protective effects of E_2 have been documented in numerous animal studies but have also been reported in humans as well [24-30,84,85]. It has been suggested that the decline in production of ovarian hormones contributes to the increased incidence of stroke in postmenopausal women [24,86]. In fact, a number of observational and epidemiological studies have reported that E_2 replacement therapy in postmenopausal women reduces stroke as well as

coronary heart disease and neurodegenerative diseases [87-93]. Importantly, recent analysis of data from the Women's Health Initiative indicates that women who initiate E_2 replacement therapy before 60 years of age or within ten years of menopause have reduced rates of coronary heart disease and mortality [84,85].

Taken together, our studies demonstrate that E_2 treatment increases Ape1 expression in the cerebral cortex and suggest that E_2 mediates its neuroprotective effects in the brain in part by increasing expression of oxidative stress response proteins and reducing oxidative DNA damage. Because the maintenance or overexpression of Ape1 results in decreased ischemia-induced damage [16-18] and the decline of Ape1 expression leads to an increase in ischemic damage [16,22,23], we believe that the E_2-mediated increase in Ape1 expression enables neurons to repair DNA lesions, reduce oxidized cellular proteins, and more effectively regulate transcription.

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Figures and Figure Legends

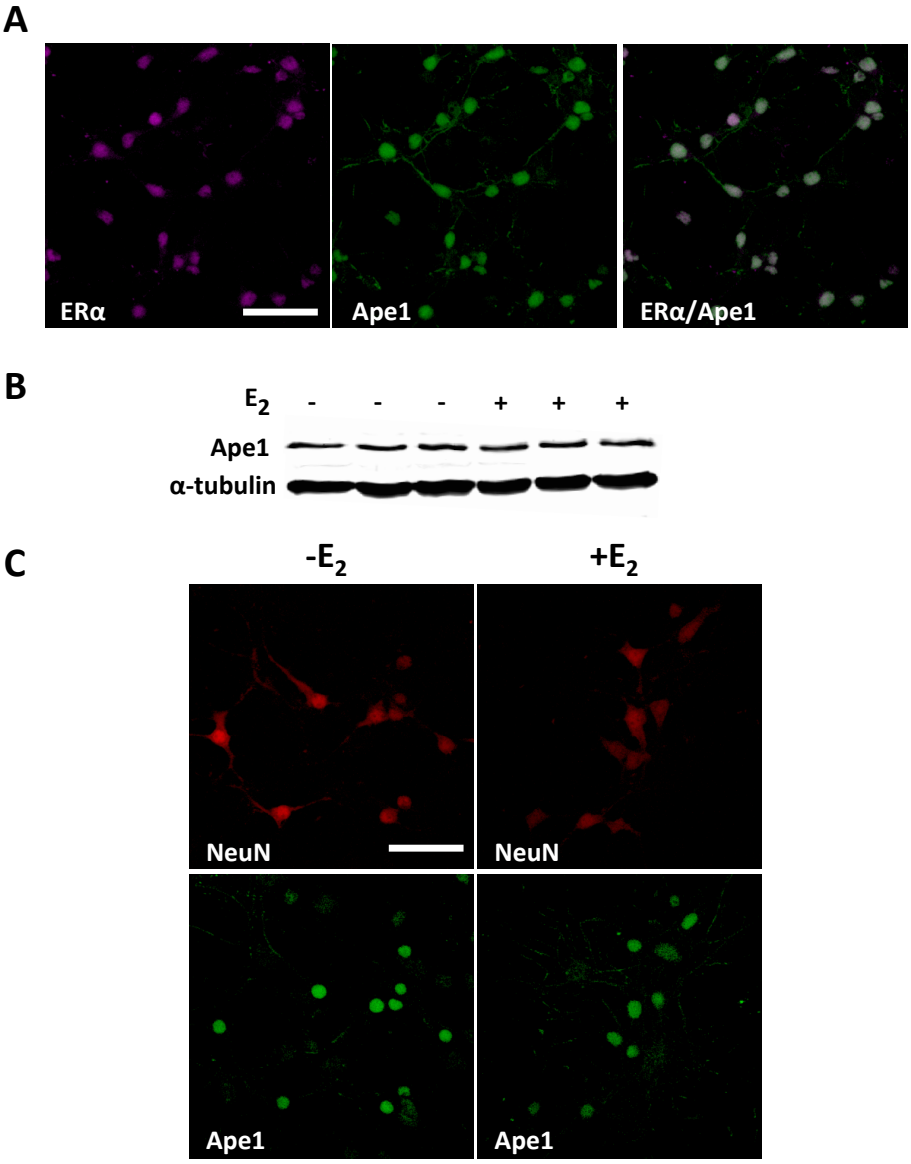

Fig. 3.1 Ape1 and ERα are expressed in primary cortical neurons. Cerebral cortical neurons were isolated from P0 mouse pups and cultured in vitro for 10 days. (A) Immunofluorescent staining was performed using ERα- and Ape1-specific antibodies. Neurons were treated with ethanol or 20 nM E_2 for 24 h and whole cell extracts were prepared for (B) Western blot analysis using Ape1- and α-tubulin-specific antibodies or (C) immunofluorescent staining using Ape1- and NeuN-specific antibodies. Scale bars indicate 25 μm.

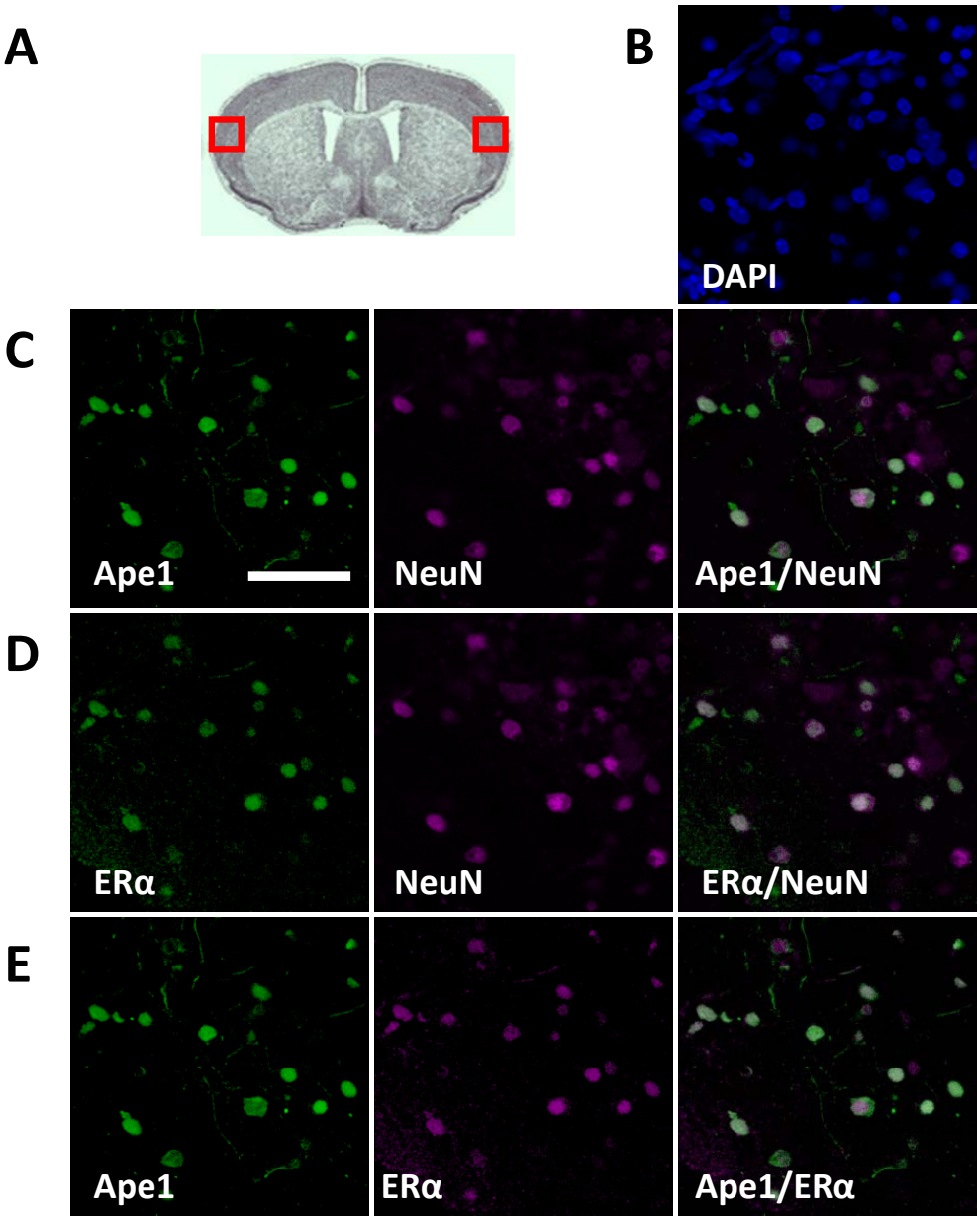

Fig. 3.2 Ape1 and ERα are expressed in cortical neurons in brain slice cultures. (A) Red boxes indicate the regions of the cerebral cortex examined by immunofluorescence. Immunofluorescent staining was performed with brain slice cultures using (B) DAPI to identify cortical cell nuclei, (C) Ape1- and NeuN-, (D) ERα- and NeuN-, or (E) Ape1- and ERα-specific antibodies. Scale bar indicates 25 μm.

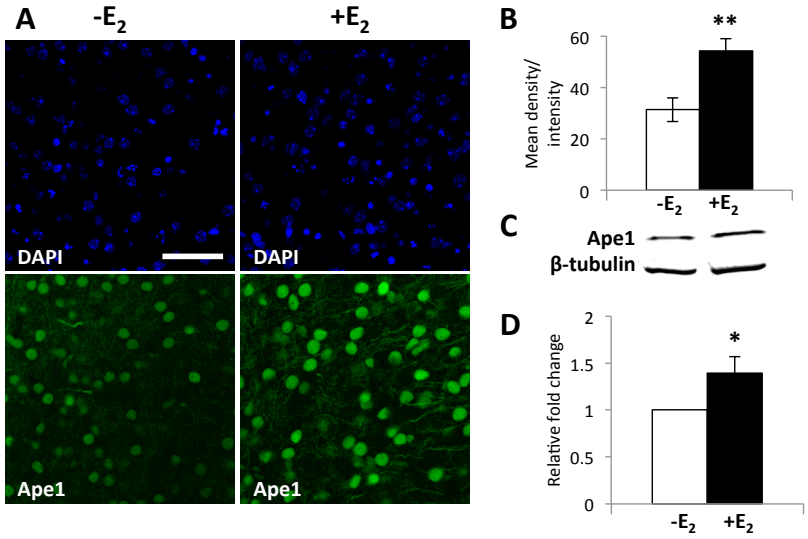

Fig. 3.3 E_2 increases Ape1 expression in brain slice cultures. Brain slice cultures were treated with ethanol or 20nM E_2 for 24 h. (A) Immunofluorescent staining was performed using an Ape1-specific antibody. DAPI staining was included to identify cortical cell nuclei. Scale bar indicates 25 μm. (B) Image analysis of Ape1 expression from 6 individual ethanol- or 7 individual E_2-treated brain slice cultures were combined and are expressed as the mean density/intensity ± SEM. (C) Whole cell extracts from brain slice cultures were prepared and quantitative Western blot analysis was performed using Ape1- and β-tubulin-specific antibodies. Ape1 expression was normalized to β-tubulin expression. (D) Data from 8 individual brain slice cultures were combined and are expressed as the normalized expression ± SEM. Ape1 expression from ethanol- or E_2-treated brain slice cultures were compared using Student's *t* test to determine statistical significance (* $p < 0.05$, ** $p < 0.001$).

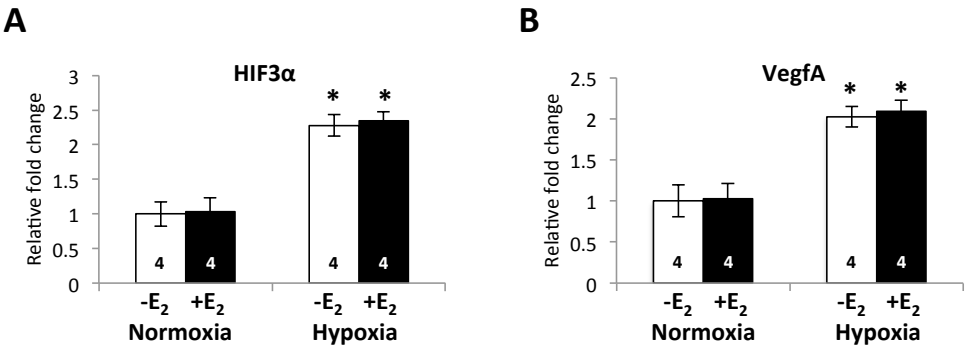

Fig. 3.4 Hypoxia significantly increases HIF3α and VegfA transcript levels. Ovariectomized female mice were treated with oil or E_2 for 7 days and subjected to normoxic or hypoxic conditions. Cortices were dissected and total RNA was isolated. cDNA was synthesized and quantitative real-time PCR was carried out with (A) HIF3α- or (B) VegfA-specific primers. The relative fold change was calculated using the delta-delta Ct method with ribosomal protein L7 (RPL7) as a control. The mean relative fold change in each group is shown ± SEM. Two-way analysis of variance (ANOVA) was used to detect a significant difference in mRNA levels from mice that had been exposed to hypoxia compared with mice that had been exposed to normoxia (* $p < 0.0001$). The number of animals in each treatment group is indicated at the base of each bar.

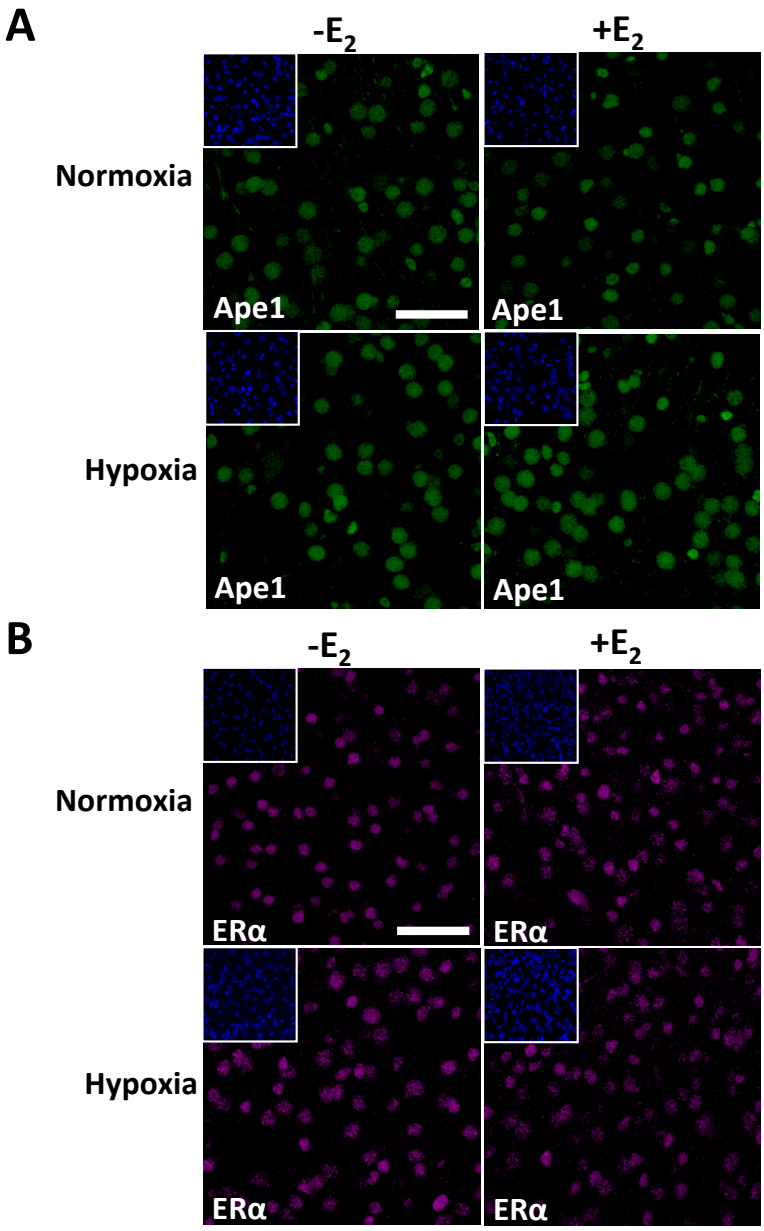

Fig. 3.5 E_2 increases Ape1 expression after hypoxia in the mouse cerebral cortex.
Ovariectomized female mice were treated with oil or E_2 for 7 days, subjected to normoxic or hypoxic conditions, and allowed to recover for 3h. Immunofluorescent staining was performed using an (A) Ape1- or (B) ERα-specific antibody. DAPI staining is shown in the inserts. Scale bars indicate 25 μm.

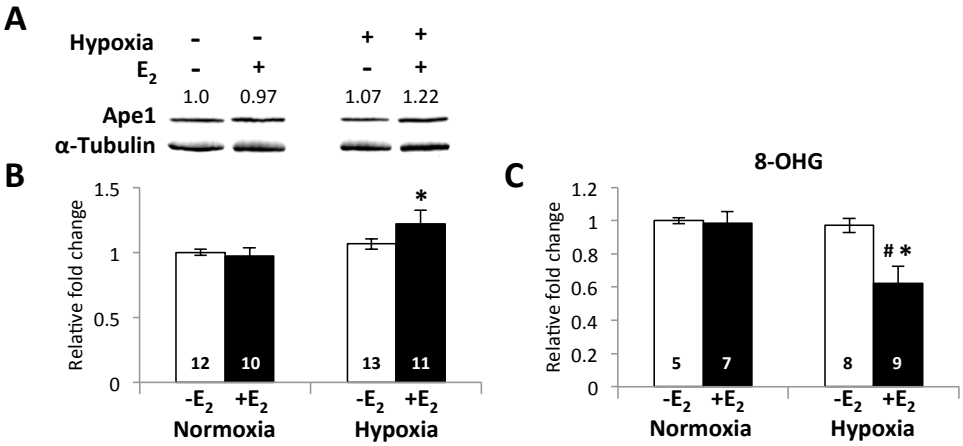

Fig. 3.6 E_2 and hypoxia increase Ape1 protein expression and decrease 8-OHG damage in the mouse cerebral cortex. Ovariectomized female mice were treated with oil or E_2 for 7 days and subjected to hypoxic conditions. (A) Quantitative Western blot analysis using whole cell extracts from cerebral cortices was performed using Ape1- and α-tubulin-specific antibodies. (B) Ape1 expression was normalized to α-tubulin expression and data are expressed as the relative fold change ± SEM. The value above each band indicates the relative fold change for each condition. (C) Genomic DNA was isolated from each cerebral cortex and 8-OHG concentrations were measured. Data represent the normalized mean ± SEM. Two-way analysis of variance (ANOVA) was used to detect significant differences in Ape1 protein expression or 8-OHG level in response to hypoxia (* $p < 0.05$) or E_2 (# $p < 0.05$). Relative fold change on the Y-axis indicates the normalized mean value for each treatment with the value of oil-treated mice maintained at normoxic conditions set at 1. The number of animals in each treatment group is indicated at the base of each bar.

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CHAPTER FOUR:

Estrogen Receptor α Protein Expression in the Cerebral Cortex

In preparation

Abstract

Although estrogen receptor alpha (ERα) and 17β-estradiol play critical roles in target tissues, little is known about the expression of ERα in the cerebral cortex. We have examined ERα mRNA and protein levels in the cerebral cortex of female mice at postnatal days 5 and 17 and at 4, 13, and 18 months of age. We found that although ERα transcript levels declined from postnatal day 5 through 18 months of age, ERα protein levels remained stable. Importantly, E_2- and ERα-regulated expression of the progesterone receptor gene demonstrated that, not only is ERα present, but it is functional in younger and in older females suggesting that age-related changes in estrogen responsiveness in the cerebral cortex are not due to the absence of ERα protein.

1. Introduction

17β-estradiol (E_2) is a steroid hormone that regulates gene expression in the reproductive tract as well as non-reproductive tissues including the cardiovascular, skeletal, and nervous systems [1-3]. These tissues respond to E_2 in part because they express the estrogen receptor (ER). ERα and ERβ are members of the steroid hormone receptor superfamily and are responsible for mediating the classical, genomic responses to E_2 in target tissues [4].

In the brain, E_2 regulates sexual development and differentiation, synaptogenesis, learning and memory, mood, auditory perception, and neuroprotection [5-9]. The regions of the brain that have been reported to respond to E_2 include the hypothalamus, hippocampus, amygdala, central auditory system, and cerebral cortex [3,5,10].

ERα has several vital functions in the cerebral cortex. For example, it is important for neurogenesis [11] and protecting the cerebral cortex from ischemia-induced injury [8,12]. ERα also plays an important role in executive functioning of the prefrontal cortex which includes working memory, attention, and behavioral inhibition [13].

Previous studies have examined ERα expression in the cerebral cortex [14-21]. ERα mRNA is highly expressed in the cerebral cortex shortly after birth but then decreases sharply beginning at postnatal day 10 (P10) [14-16]. ERα mRNA has also been detected in the cerebral cortices of aged female mice [17]. However, it has become clear that mRNA levels may not accurately reflect protein levels [22,23]. Thus, studies that use mRNA data to predict relative protein levels must be interpreted with caution.

The studies that have examined the expression of ERα protein in the cerebral cortex have done so at one or two time points [15,18-21]. For example, Merchenthaler and coworkers used autoradiographic and immunohistochemical analyses to examine the distribution of ER protein in the adult female mouse brain [20]. This study demonstrated that the receptor was present at low levels in the cerebral cortex in 60-90 day old mice. While this study examined the spatial distribution of ERα in the cerebral cortex in great detail, it included only a single time point. Any alterations that occurred over time would not have been observed.

In the current studies, the temporal pattern of ERα mRNA and protein expression in the cerebral cortex was followed over 18 months in hormonally intact female mice. Our work demonstrates that cortical ERα protein expression remains stable in these mice and that the ERα

protein present is functional suggesting that age-related changes in estrogen responsiveness in the cerebral cortex are not due to the absence of ERα protein.

2. Materials and methods

2.1. Mice

C57BL/6 breeding pairs for the generation of mouse pups (P5 and P17) and adult females (4 months) were obtained from Jackson Laboratory (Bar Harbor, ME). Retired female breeders were housed until 13 months for use as middle age animals. 18 month old C57BL/6 females were purchased from the National Institute on Aging. All mice were maintained on a 12 h light/dark schedule with access to water and food ad libitum. All procedures were performed in accordance with guidelines of the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee and Division of Animal Resources.

2.2. Estrous cycle staging

4 month, 13 month, and 18 month old female mice were monitored for estrous cyclicity using vaginal cytology. Briefly, 5 μL of PBS was inserted into the vagina of each mouse and then removed, spread on a glass slide, allowed to air dry, fixed, and then nuclei were stained (3300-APD, Richard Allan Scientific, Pittsburgh, PA). Slides were imaged at 400x magnification using bright field illumination and the Leica DM 4000 B microscope. The stage of the estrous cycle was based on the presence or absence of leukocytes, cornified epithelial, and nucleated epithelial cells [24,25]. 4 month old mice exhibited at least two regular estrous cycles, the 13 month old mice had irregular estrous cycles, and the 18 month old mice were in persistent diestrus. 4 month, 13 month, and 18 month old female mice were sacrificed while in diestrus.

2.3. RT-PCR

Mice were anesthetized by inhalation of isoflurane and decapitated. Total RNA was isolated from cortices using RNAqueous reagents (Ambion, Life Technologies, Austin, TX) according to the manufacturer's instructions. RNA concentrations were measured and cDNA was synthesized using the iScript kit (Bio-Rad, Hercules, CA) as described by the manufacturer. 2 μl of cDNA was combined with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), forward (5'-AGTGTCTGTGATCTTGTCCAG-3') and reverse (5'-TGTGTGCCTCAAATCCATCA-3') primers for ERα that spanned exons 7 and 8, forward (5'-GGTGAGGCCGGTGCTGAGTATG-3') and reverse (5'-GACCCGTTTGGCTCCACCCTTC-3') primers for GAPDH, or forward (5'-CCGCCATACCTTAACTACCTGAG-3') and reverse (5'-TGCTGCCCTTCCATTGCC-3')

primers for PR and real-time PCR was carried out using a Bio-Rad iQ5 multicolor Real-Time PCR Detection System. Standard curves were created using cDNA equivalents of 0.25, 2.5 and 25 ng of RNA and were run in triplicate with each primer set for each experiment.

2.4. Immunofluorescence imaging

Wild type P5, P17, 4 month, 13 month, and 18 month-old female mice were anesthetized by inhalation of isoflurane and decapitated. Whole brains were quickly harvested, bisected sagittally, and fixed in 4% paraformaldehyde for 18-24 h. Tissue was then transferred to a Leica ASP300 tissue processor for dehydration, clearing, and paraffin infiltration. Tissue was embedded in paraffin and four-micrometer coronal sections were mounted on charged glass slides. Tissue sections were deparaffinized in xylene, rehydrated in two changes of 100% then two changes of 95% ethanol, and washed with PBS. Sections were then heated in 10 mM citric acid, pH 6.0, for 20 min for antigen retrieval and remained in the citric acid until the temperature reached ~30°C (~2 h). The sections were then washed 3x with PBS, incubated in blocking buffer (PBS with 0.05% Tween-20 and 5% normal donkey serum) for 10 min, and incubated in blocking buffer with an ERα- specific antibody (1:600, ab31312, Abcam Inc., or 1:50, sc542, Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C. The next day sections were washed 3x with PBS containing 0.1% Tween-20 (PBS-T). Then sections were incubated with DyLight 649-conjugated anti-rabbit IgG (1:500, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 30 min in the dark at room temperature, washed 3x with PBS-T, incubated with 4′,6-diamidino-2-phenylindole (1:1000, DAPI) nucleic acid stain for 10 min at room temperature, washed 3x with PBS, and mounted with Pro-Long Gold antifade reagent (Life Technologies, Grand Island, NY). DAPI co-staining was included for each treatment to identify nuclei and ensure that similar numbers of cells were present. Control slides, which had not been exposed to primary antibody, and tissue sections from ERα knockout mice were processed and run in parallel. As an additional negative control, the ERα- specific antibody ab31312 was incubated with ten molar fold excess of purified, full-length human ERα protein prior to incubation of the antibody on brain tissue.

2.5. Immunofluorescent image collection and quantitation

All images were obtained with a 40x oil-immersion objective using a Leica DM 4000 B confocal microscope and imaging was performed using the Leica TCS SPE system and Application Suite Advanced Fluorescence software (Leica Microsystems, Inc., Bannockburn,

IL). Detector gain and offset, laser power, and bandwidth of emission collection were kept constant for all treatments in each experiment and adjusted so that images had a full range of pixel intensities (0–255) and saturation was minimized.

Quantitative immunofluorescent analysis of ERα staining in brain tissue was performed by using DAPI staining to count the total number of cells in 3-6 fields from 3-4 mice. The percent of cells expressing ERα was determined.

2.6. Immunohistochemistry

Immunohistochemistry was performed as described for immunofluorescence except that after antigen retrieval endogenous peroxidases were blocked with 3% hydrogen peroxide for 20 min at room temperature. After three 5 min washes in PBS, sections were incubated in blocking buffer for 10 min and then incubated in blocking buffer with a PR-specific antibody (1:50, A0098, Dako, Carpinteria, CA) overnight at 4°C. Sections were washed with PBS-T 3x for 5 min each and then incubated with biotin-conjugated secondary antibody (1:200, Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature, followed by three 5 min washes with PBS-T. The ABC Peroxidase Staining kit (1:100 dilution of each Reagent A and B in PBS, 32020, Thermo Scientific, Rockford, IL) was applied to the sections for 30 min. After three washes with PBS, staining was visualized with peroxidase-sensitive Sigmafast 3,3'-Diaminobenzidine tablets (DAB, Sigma, St. Louis, MO) for 10 min. Sections were counterstained with 0.1% methyl green (Sigma, St. Louis, MO) for 5 min at 60°C, dehydrated in ethanol, cleared in xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Control slices, which had not been exposed to primary antibody, were processed in parallel. Images were obtained at 40x using a Leica DM 4000 B confocal microscope with the Retiga 2000R digital camera and Image Pro Plus image collection and analysis software.

2.7. Statistics

Combined data are expressed as the mean ± SEM. SAS version 9.3 (SAS Institute Inc., Cary, NC) was used for statistical analysis. ERα and PR transcript levels and the percent of ERα-positive cells were compared using one-way analysis of variance (ANOVA) and Tukey's post-hoc test. A p value of <0.05 was considered statistically significant (95% confidence interval).

3. Results

3.1. ERα transcript levels in the cerebral cortex throughout the female lifespan

Much of the previous work examining ERα expression in the cerebral cortex has analyzed ERα transcript levels in rodents either shortly after birth or in adults [14-17]. Therefore, to examine ERα expression across a broad range of ages in a single study, we measured ERα mRNA at five time points in hormonally intact females. We noted a significant decline in ERα transcript levels after P5 (Fig. 4.1), which is in agreement with previous work [15]. Another significant decrease occurred at 13 months and was sustained through 18 months. These results demonstrated that ERα mRNA levels decline with age in intact female mice, most notably after P5, and is consistent with data from previous studies [14-16,26]. However, in contrast with previous studies in which ERα mRNA levels were very low at P25 [15,16,26] and another study in which ERα mRNA was not detected in young (3-4 month) female mice [14], our work demonstrates that ERα mRNA transcripts were detected at each of the ages examined.

3.2. ERα protein expression in the cerebral cortex throughout the female lifespan

While mRNA levels provide valuable information, protein, not mRNA, is the active end product of gene expression. Therefore, ERα protein expression was examined in the cerebral cortex of female mice using immunofluorescence at various ages. ERα protein was clearly seen in the cerebral cortex of female mice at P5 (Fig. 4.2A). While the DAPI staining suggests that the number and density of cells in the cortex had decreased by P17 (Fig. 4.2B), this synaptic pruning and subsequent cell death is a normal occurrence during the first two weeks of postnatal brain development [27-30], ERα protein expression continued to be detected at high levels in the cerebral cortex. 4 month old female mice (Fig. 4.2C) expressed similar levels of ERα protein. Even in middle age (13 months, Fig. 4.2D) and aged (18 month, Fig. 4.2E) mice, ERα protein expression was clearly present in the cerebral cortex.

Image analysis of cerebral cortices from female mice at P5, P17, 4 months, 13 months, or 18 months indicated that 81%, 77%, 72%, 67%, or 70% of the total DAPI-stained cells expressed ERα, respectively (Fig. 4.2F). While a slight decrease in the total percent of cells expressing ERα was observed over time, this decline was not statistically significant. These results demonstrate that ERα protein expression remained high in the cerebral cortex of hormonally intact females, even in middle age and aged mice.

3.3. Anti-ERα antibody recognized ERα protein in the cerebral cortex

Since ERα protein levels were very different from the ERα transcript levels, we wanted to verify the specificity of our ERα primary antibody. Figure 4.3A demonstrates the typical staining pattern and intensity for ERα that was observed when the cerebral cortex was incubated with primary and secondary antibodies. However, when the anti-ERα primary antibody (ab31312) was incubated with purified, full-length human ERα protein and then added to brain slices from the same mouse, no staining was observed (Fig. 4.3B). When the primary antibody was omitted and only the secondary antibody was used, no staining was observed (Fig. 4.3C).

As final evidence that the anti-ERα primary antibody was specific, brain slices from ERα knockout or wild type mice were stained. As shown in figure 4.3D, staining was only observed in wild-type mice. Thus, our antibody was specific for ERα and did not recognize other epitopes.

3.4. Expression of an ERα-regulated gene in the cerebral cortex

Our studies thus far have demonstrated that ERα mRNA and protein were present in the cerebral cortex of mice from P5 through 18 months of age. To investigate whether ERα was functional at each of the ages examined, we evaluated mRNA and protein expression levels of the E_2-responsive gene progesterone receptor (PR) in the cerebral cortex of hormonally intact female mice at various ages. PR mRNA expression declined slightly, but not significantly, from P5 to P17 but increased significantly in mice 4 months of age (Fig. 4.4A). In 13 and 18 month old mice, PR mRNA expression significantly declined to levels below P5. To examine PR protein levels in female mice at each age, immunohistochemistry was performed using a PR-specific antibody. Our work demonstrated that PR protein is present at P5, P17, 4 months, 13 months, and 18 months of age (Fig. 4.4B-F). Thus, while ERα protein levels remained stable throughout the lifespan of intact female mice, ERα-regulated PR mRNA diminished in the 13 and 18 month old mice and PR protein was present at each age. These results indicate that the ERα protein present in the cerebral cortex at each age was functional and influenced transcription of PR.

4. Discussion

Because ERα mediates many of the cellular responses of the cerebral cortex to E_2 such as cognition [13] and neuroprotection [8,11,12], this protein plays a critical role in neurological function. While a number of studies have examined ERα mRNA levels in the cerebral cortex,

there has been some question about whether ERα protein is present [15,18,21]. We have now demonstrated that ERα protein was present in the cerebral cortex of newborn, middle aged, and older female mice.

Although the expression of ERα has been examined in the hypothalamus and hippocampus in detail, far less is known about the expression of ERα in the cerebral cortex. Our studies focused on the cerebral cortex since this region of the brain is more susceptible to ischemic damage than other brain regions [6,7,31-35] and we were interested in determining whether ERα might be present in the cerebral cortex of older animals and capable of mediating estrogen responsiveness.

Previous studies with ERα null mice have demonstrated that PR gene expression depends on ERα expression [36-38]. We have now shown that PR expression is sustained in the cerebral cortex over 18 months suggesting that not only was ERα present, but that it was functional and was capable of enhancing PR mRNA and protein levels. Although the level of PR mRNA varied somewhat with age with the most abundant transcripts being present during peak reproductive age, immunohistochemistry suggested that E_2- and ERα-mediated expression of PR protein was sustained in younger and in older female animals.

Expression of ERα has been examined in the rodent brain using ligand binding, autoradiography, immunohistochemistry, in-situ hybridization, and PCR analyses [14-16,19,20,39,40]. While newer techniques have made it possible to distinguish between ERα and ERβ, it is clear that ERα is responsible for E_2-mediated neuroprotection [8,9]. Thus, our studies focused on ERα protein expression. In fact, ERα protein expression is sustained in the cerebral cortex suggesting that E_2 may mediate its neuroprotective effect in younger as well as older females.

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Figures and Figure Legends

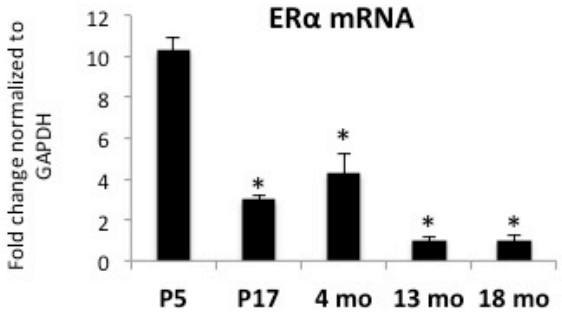

Fig. 4.1 ERα transcript levels decline in the cerebral cortex throughout the female lifespan. Total RNA was isolated from the cortex of female mice at postnatal day 5 (P5), postnatal day 17 (P17), 4 months (4 mo), 13 months (13 mo), and 18 months (18 mo) of age. cDNA was synthesized and ERα mRNA levels were determined using quantitative real time PCR. The relative fold change was calculated using the delta-delta Ct method and each sample was normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA present. The mean relative fold change at each age is shown ± SEM. One-way analysis of variance (ANOVA) and Tukey's post-hoc test was used to detect whether there were significant differences in ERα mRNA from female mice at P5 compared with mice at P17, 4 mo, 13 mo, and 18 mo (* $p < 0.05$) of age.

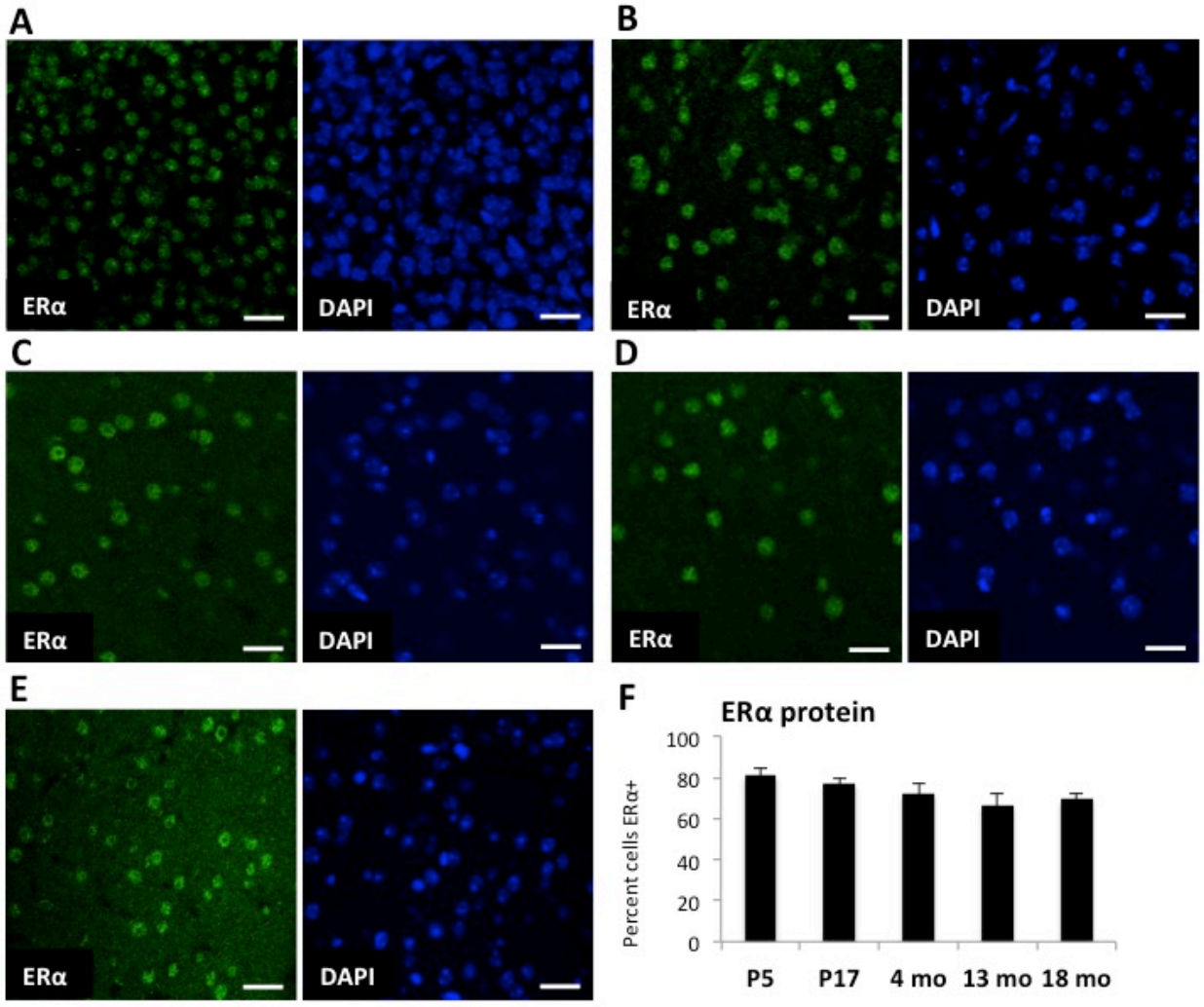

Fig. 4.2 ERα protein expression remains stable in the cerebral cortex throughout the female lifespan. Brain sections from female mice at (A) postnatal day 5, (B) postnatal day 17, (C) 4 months, (D) 13 months, or (E) 18 months of age were stained using immunofluorescence with an ERα-specific antibody and the cerebral cortex was examined. DAPI staining was included to identify cortical cell nuclei. Scale bars indicate 25 μm. (F) Quantitation of ERα protein expression was performed by determining the percent of ERα-positive cells ± SEM.

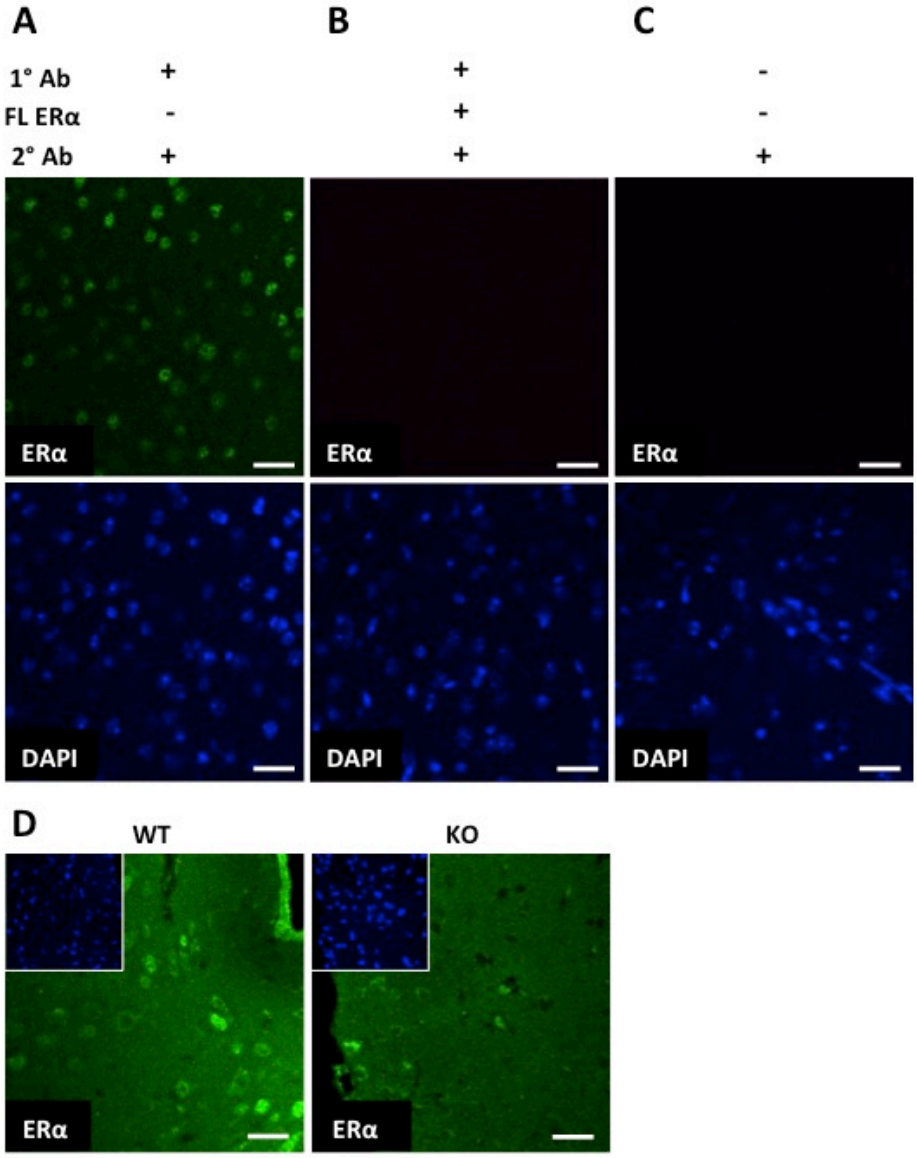

Fig. 4.3 Anti-ERα antibody recognizes ERα in the cerebral cortex. Brain sections were obtained from 13 month old female mice and using the ERα-specific antibody ab31312 (A) was not or (B) was pre-incubated with full-length human ERα protein. (C) The ERα primary antibody was omitted so that only secondary antibody was used. (D) Brain sections from wild type (WT) or ERα knockout (KO) adult female mice were stained using the ERα-specific antibody sc542. Scale bars indicate 25 μm.

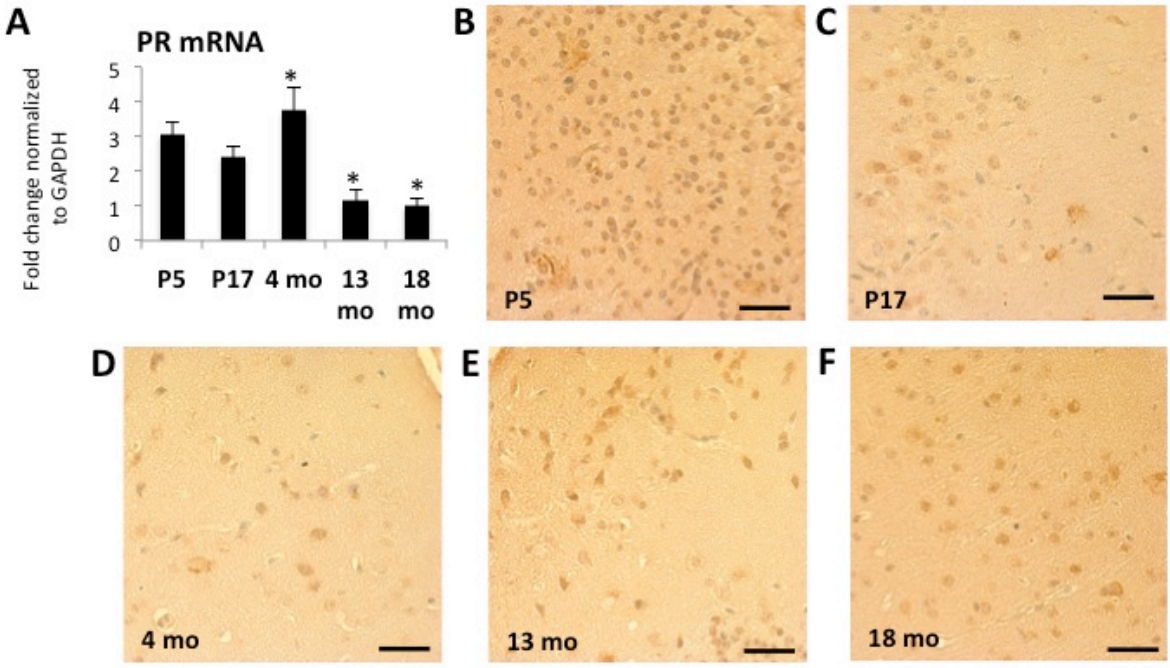

Fig. 4.4 PR transcript levels decline but PR protein is present in the cerebral cortex throughout the female lifespan. (A) Total RNA was isolated from the cortex of female mice at P5, P17, 4 mo, 13 mo, and 18 mo. cDNA was synthesized and PR mRNA levels were determined using quantitative real time PCR. The relative fold change was calculated using the delta-delta Ct method and each sample was normalized to the amount glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA present. The mean relative fold change at each age is shown ± SEM. One-way analysis of variance (ANOVA) and Tukey's post-hoc test was used to detect significant differences in ERα mRNA from female mice at P5 compared with mice at P17, 4 mo, 13 mo, and 18 mo (* $p < 0.05$) of age. Brain sections from (B) P5, (C) P17, (D) 4 mo, (E) 13 mo, and (F) 18 mo old female mice were stained using immunohistochemistry with a PR-specific antibody to examine the cerebral cortex. Scale bars indicate 25 μm.

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CHAPTER FIVE

Conclusions

Summary

The work in this dissertation reveals several novel findings. First, I used a rodent model of amyotrophic lateral sclerosis (ALS) in which Cu/Zn superoxide dismutase was mutated to examine nitrotyrosine levels in spinal cord sections and demonstrated that oxidative protein damage is an early feature of ALS that appears prior to motor symptoms. This work extends our understanding of the development and progression of protein damage in ALS and, subsequently, progression of motor symptoms. Second, I showed that ovariectomized female mice that had been treated with E_2 and exposed to hypoxia had increased protein expression of apurinic endonuclease in the cerebral cortex. We believe that this E_2-mediated increase in apurinic endonuclease expression contributes to a decline of oxidative DNA damage in the cerebral cortex of mice that had been pretreated with E_2 and then exposed to hypoxia. Finally, using hormonally intact female mice, I demonstrated that ERα protein expression in the cerebral cortex remains stable and functional up to 18 months of age. Taken together, these studies provide data that may be valuable for improving the diagnosis and outcome of oxidative stress-induced diseases.

Ligand specificity

Human studies have demonstrated that the decline in ovarian hormones following menopause contributes to an increased risk of ischemic stroke and neurodegenerative disorders in women [1,2]. One means by which a woman may overcome these debilitating symptoms associated with menopause is the administration of estrogen [3-6]. Because systemic exposure to E_2 may have unintentional side effects, the use of a brain-specific estrogen could be extremely beneficial [7]. A well-known example of one selective estrogen receptor modulator influencing ER activity in one tissue but not in another is tamoxifen. In fact, tamoxifen has been used for decades in the clinic as an antagonist of ERα to treat breast cancer while maintaining bone mineral density and improving cholesterol levels [8-11]. In the brain, selective estrogen receptor modulators have been shown to reduce infarct size, inflammation, superoxide production, oxidative DNA damage, and lipid peroxidation following ischemia but do not act as estrogen receptor agonists in breast and uterine tissue [7,12-15].

In addition to tissue specificity, ligands also exhibit receptor specificity. While E_2 binds to both ERα and ERβ, and ERβ has been implicated in E_2-mediated neuroprotection [16-18], ERα is the primary receptor responsible for mediating E_2-induced neuroprotection. This was demonstrated by a reduced infarct volume in ERβ-, but not in ERα-knockout mice that had been ovariectomized, exposed to E_2, and then subjected to middle cerebral artery occlusion [19-21]. Additionally, the ERα-selective agonist propyl pyrazole triol (PPT) is neuroprotective in a rodent model of Parkinson's disease and deletion of ERα increases the risk of developing Parkinsonian symptoms [22-24]. However, pretreatment with the ERβ-selective agonist diarylpropiolnitril (DPN) did not decrease infarct size or improve sensorimotor function in rats following middle cerebral artery occlusion [25]. These studies demonstrate the importance of ERα in protecting the brain from ischemic damage and other neurodegenerative insults and highlight its role as the primary ER mediating these effects in the brain.

Global analysis

While the work in this dissertation provides valuable information and details about mechanisms involved in E_2-mediated neuroprotection, a more global and unbiased approach to elucidate mechanisms regulating E_2-mediated neuroprotection is RNA sequencing technology [26]. In fact, studies in our laboratory using RNA sequencing are underway to uncover pathways and additional genes that are altered in the cerebral cortex by E_2 and hypoxia. Initial findings suggest that E_2 regulates many genes in the cerebral cortex that had previously not been identified as E_2-responsive in the brain. Biological processes and pathways associated with E_2-regulated genes include chromosome organization, gap junction and cell adhesion molecules, inflammation, and MAPK signaling.

Conclusions

The metabolic needs of the brain are the greatest of any organ in the body and this results in the greatest production of ROS. By understanding the protein and DNA damage that results from ROS exposure, our work provides important data that can be used to decrease this ROS-induced damage in the brain. Clinical applications that utilize this information may result in treatments that slow the progression and improve the outcome of diseases such as ALS and ischemia. The need for improved, tissue-specific therapies that can protect women from ischemic stroke and other neurodegenerative disorders without detrimental side effects is even more pressing than ever.

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